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POTENTIATION OF TETRACYCLINE ANTIBIOTICS WITH TEREPHTHALIC ACID AND LOW DIETARY CALCIUM

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RECENTLY there has been considerable interest in the potentiation of antibiotics. The potentiation of tetracycline antibiotics is not new. This interest was stimulated by a report of Peterson.[3] Potentiation results in increased serum levels of antibiotics and thus additional clinical therapeutic activity. Within the scope of this paper, potentiation is limited to the oral use of tetracycline antibiotics.

If potentiation on a commercial scale is possible, there are at least these two distinct advantages: (1) in some diseases it would be possible to obtain beneficial results by the use of lower levels of antibiotic and thus treatment could be attained with less cost, (2) the use of potentiated antibiotic feeds would allow increased antibiotic activity by using the same amount of the medicament. In the first instance, for example, infectious synovitis could be just as effectively treated by the use of 100 grams of a tetracycline antibiotic per ton of feed if potentiated, as by 200 grams unpotentiated. In the latter instance, potentiation could enhance the bene-

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ficial results in certain diseases where 200 grams may not be an optimum dose level. Recent publications by Price and Zolli[4,5] indicate that the use of terephthalic acid (TPA) at the 0.5 percent level in the feed increases the serum level of a tetracycline antibiotic two to three fold. Our research with TPA and also with low calcium diets was designed to check these findings. In our experimental work on the control of disease, infectious synovitis was used as an indicator of antibiotic activity.

EXPERIMENTAL

Experiment 1. The comparison of terephthalic acid and a low calcium diet as potentiators of chlortetracycline.

The chickens used in this experiment were of the Delaware strain and were hatched on the University of Delaware farm. Each treatment group per pen in this experiment consisted of three birds. The total weight of each pen was taken at the start of the treatment period. The serum level of chlortetracycline in each chicken was determined prior to and at the end of the treatment period of 5 days. The antibiotic levels were determined by the method prescribed by the Pure Food and Drug Administration. Serum calcium levels were determined by the method of Clark and Collip.[2] A list of the various treatments and the results obtained are shown in table 1. TPA, when used, was added at the rate of 0.5 percent. The low calcium diet was prepared by eliminating the calcium phosphate, bone meal and fish meal from a standard commercial broiler diet. The high phosphorus diet was prepared by adding disodium acid phosphate. The low calcium diet and the high phosphorus diet were assayed for these two minerals by a commercial feed analysis laboratory.

The results (table 1) indicate that the TPA, the low calcium and the high phosphorus diets increased the serum levels of antibiotic since the unpotentiated diet did not produce detectable serum levels. The 200 gram level of chlortetracycline, under the conditions of our tests, does not always produce a detectable serum level.

As a second part of this experiment, feed levels of 25, 50, 100, 200 and 300 grams of chlortetracycline were fed to 8-

week-old chickens for 5 days. Parallel levels were given to chickens with TPA added at the 0.5 percent level. Three birds were placed on each treatment and serum samples were taken on the 5th day of treatment. The serum levels of each group (average of three birds) are shown in table 1 A. Definite potentiation was produced by the TPA.

TABLE I

Serum level of antibiotics in birds on potentiated and non-potentiated diets

Pen	At start total wgt. in lbs.*	Increase weight in lbs.*	Feed treatment	Serum level** of antibiotics 5th day*	Mg./100 ml. serum calcium*
1	4.75	0.35	Regular Feed	None	5.5
2	4.25	0.45	200 Grams Aureo/ton	None	7.0
3	4.60	0.25	200 Grams Aureo/ton + TPA	.008	6.50
4	4.70	0.25	200 Grams Aureo/ton + low Ca	.011	6.29
5	4.65	0.45	200 Grams Aureo/ton + Hi Phos.	.006	6.40

* Average for 3 birds.
Treatment: TPA = 0.5%
Low Ca = 0.7% by chemical assay
Hi Phos. = 1.90% ($Na_2 HPO_4$ added) by chemical assay
** Serum level of antibiotic = mcg. per ml.

TABLE I A

Serum levels of chlortetracycline following TPA potentiation of feed

Level of chlortetracycline	Serum level* unpotentiated	Serum level 0.5% TPA
25 gms per ton	0	.0
50 gms per ton	0	.014
100 gms per ton	0	.143
200 gms per ton	.10	.202
300 gms per ton	.141	.202

* All serum levels average of 3 birds and expressed as mcg per .2 ml.

Experiment 2. A second experiment of similar design was also completed. The various treatments and experimental results are indicated in table 2. In this experiment the results are similar to those of the first with the exception that the high phosphorus diet did not produce a detectable serum level of the antibiotic.

Experiment 3. In order to test potentiation under field

conditions, the disease infectious synovitis was used as an indicator of biological activity. Since the dose level of chlortetracycline for the prophylactic and therapeutic treatment of this disease has been definitely established, this method of testing for potentiation was considered to be feasible. The experimental disease was produced by injecting the birds intramuscularly with 0.5 ml of a culture of infectious synovitis (isolate #96). A total of 20 pens of 300 birds each were involved in the experiment. The preventative treatments were started at the time of disease inoculation and the therapeutic treatments were started 5 days later when the birds were just showing signs of the disease. TPA when used, was added to the diet at the level of 0.5 percent. The pen distribution of the treatments and the results are shown in table 3. The prevailing price of TPA was 40 cents per pound. The cost of 200 grams of chlortetracycline at the rate of 8 cents a gram was $16.00. The cost of the treatment consisting of 100 grams of chlortetracycline with 0.5 percent TPA was $12.00. It will be noted that 100 grams of chlortetracycline with TPA controlled the infection as effectively as 200 grams per ton of chlortetracycline alone.

Experiment 4. This experiment was similar to the preceding one. Three hundred Arbor Acre X Vantress birds were placed in each pen and injected intramuscularly with 0.5 ml of the infectious synovitis agent at 4 weeks of age. The treatments were started 5 days later when the signs of the disease became apparent and were continued for 14 days. At this time the disease appeared to be under control. The chemical TPA was used to potentiate the tetracycline antibiotics, chlortetracycline and oxytetracycline. Two levels of TPA were tested, 0.4 percent and 0.5 percent. The results are shown in table 4. It is to be noted that the potentiated 100 gram levels were comparable to the 200 gram antibiotic levels unpotentiated.

Experiment 5. This experiment was designed to test the potentiation with a low calcium diet against infectious synovitis. The birds were injected intramuscularly at 4 weeks of age with 0.5 ml of the infectious synovitis agent. The treat-

TABLE II

Serum levels of antibiotics in birds on potentiated and non-potentiated diets

Pen	At start total wgt. in lbs.*	Increase weight in lbs.*	Feed treatment	Serum level of antibiotic at end*	Mg./100 ml. serum calcium*
1	5.1	0.50	Regular feed	None	5.58
2	5.1	0.55	200 aureo	None	6.28
3	5.1	0.30	200 aureo + TPA	0.024	6.48
4	5.6	0.60	200 aureo + low ca.	0.014	6.78
6	5.45	0.45	200 aureo + high phos.	None	6.56

* Average for 3 birds.

TABLE III

The results of TPA potentiation of chlortetracycline when tested by the control of infectious synovitis

Pen no.	Treatment	Disease	Average wt. at 6 wks.	Percent mortality	No. of unsaleable birds
1	No treatment	No disease	1.93	2.3	0
19	No treatment	No disease	1.92	0.0	0
8	No treatment	+ disease	1.83	77.3	8
15	No treatment	+ disease	1.77	91.0	5
2	Terephthalic acid	No disease (P)	1.96	0.6	0
20	Terephthalic acid	No disease (P)	2.08	1.3	0
5	Terephthalic acid	+ disease (P)	1.65	92.6	13
17	Terephthalic acid	+ disease (P)	1.62	89.0	10
6	200 gms. aureo per ton	+ disease (T)	1.81	21.3	30
14	200 gms. aureo per ton	+ disease (T)	1.69	10.6	8
9	100 gms. aureo per ton	+ disease (T)	1.65	44.0	45
13	100 gms. aureo per ton	+ disease (T)	1.78	49.0	20
3	100 gms. aureo per ton +terephthalic acid	+ disease (T)	1.76	8.0	5
18	100 gms. aureo per ton + terephthalic acid	+ disease (T)	1.66	25.3	21
10	50 gms. aureo per ton + terephthalic acid	+ disease (T)	1.60	55.6	37
16	50 gms. aureo per ton + terephthalic acid	+ disease (T)	1.56	57.3	28
4	50 gms. aureo per ton + terephthalic acid	+ disease (P)	1.76	20.0	15
11	50 gms. aureo per ton + terephthalic acid	+ disease (P)	1.77	24.3	20
7	25 gms. aureo per ton + terephthalic acid	+ disease (P)	1.56	28.6	28
12	25 gms. aureo per ton + terephthalic acid	+ disease (P)	1.76	56.6	25

P = given prophylactically at time of disease challenge.
T = given therapeutically at time signs of disease appear.
Aureo = aureomycin (R) chlortetracycline.

ments were started 6 days after inoculation when the signs of the disease became apparent and they were continued for 8 days. There were 300 Arbor Acre x Vantress birds in each pen. The results shown in table 5 indicate that treatment with 100 grams of either oxytetracycline or chlortetracycline per ton with low calcium was equivalent to the respective 200 gram per ton level of the same antibiotic unpotentiated.

TABLE IV

The control of infectious synovitis by chlortetracycline and oxytetracycline with TPA potentiation

Pen no.	Treatment	Average weight	Feed efficiency	% mortality
20	No disease control	2.81	2.73	.00
7	Disease control	2.70	3.13	22.40
8	Chlortetracycline 100 gms/T	2.94	2.81	2.00
18	+ TPA .5%	2.97	2.75	1.00
9	Chlortetracycline 100 gms/T	2.90	2.77	1.70
19	+ TPA .4%	2.77	2.95	1.40
10	Chlortetracycline 200 gms/T	2.94	2.89	1.70
17		2.96	2.82	.00
11	Oxytetracycline 100 gms/T	2.76	2.87	2.70
14	+ TPA .5%	2.68	2.93	3.05
12	Oxytetracycline 100 gms/T	2.80	2.81	1.00
15	+ TPA .4%	2.78	2.97	2.00
13	Oxytetracycline 200 gms/T	2.90	2.93	2.70
16		2.92	2.76	.67

Experiment 6. This experiment was designed to test the effect of a low calcium diet feed level to potentiate oxytetracycline when used prophylactically in turkey sinusitis. The development of the sinus lesions and the serological titre to PPLO was observed. Five 12-week-old Peterson's white turkeys were used for each treatment. The treatments used were as follows: (1) 300 grams per ton of feed of oxytetracycline, (2) 300 grams per ton of feed of oxytetracycline in a low calcium diet, (3) no medicament, (4) no medicament non-infected control. The turkeys were exposed to the disease by

the injection of 0.5 ml of infective sinus exudate. All treatments were started at the same time as the inoculation of the agent, and were continued for 21 days. The Connecticut PPLO serum plate antigen was used to determine PPLO agglutination. A serum was considered to be positive if it showed complete agglutination in a 1 to 20 dilution or higher. The serum from all turkeys in the experiment were tested prior to the inoculation of the disease agent and were found to be negative.

TABLE V

The control of infectious synovitis by potentiation with a low calcium diet

Pen no.	Treatment	Average weight	Feed efficiency	% mortality	No. unsaleable
1	Low Calcium—Neg.	2.64	2.42	1.7	0
19	Controls	2.69	2.35	.00	16
2	Reg. Feed—Neg.	2.56	2.58	5.9*	0
20	Controls	2.64	2.43	1.0	11
4	Low Calcium—Disease	2.17	4.85	59.4	29
17	Controls	2.45	4.10	51.4	53
3	Reg. Feed—Disease	2.26	4.53	52.4	34
18	Controls	2.29	4.37	59.4	68
6	Reg. Feed + 200 gms/T	2.87	2.78	17.5	66
15	Chlortetracycline	2.63	2.52	19.6	53
5	Reg. Feed + 200 gms/T	2.50	2.74	23.0	26
16	Oxytetracycline	2.45	2.86	26.2	59
10	Low Calcium + 100 gms/T	2.51	2.48	11.5	36
11	Chlortetracycline	2.52	2.69	15.7	43
9	Low Calcium + 100 gms/T	2.31	3.21	28.7	79
12	Oxytetracycline	2.53	2.85	23.8	55
8	Low Calcium + 50 gms/T	2.49	2.81	23.4	83
13	Chlortetracycline	2.57	2.89	28.3	36
7	Low Calcium + 50 gms/T	2.70	3.25	38.5	47
14	Oxytetracycline	2.53	3.86	53.8	38

* Mortality in Pen 2 due mostly to CRD.
Reg = Commercial Broiler Feed

The geometric rate of sinusitis and aerosacculitis was determined by observation of the live bird and by autopsy of birds at the end of the treatment period. The results, shown

in table 6, indicate that both the clinical and the serological response was less in the turkeys on the potentiated diet. The geometric rate of both sinuses was taken in order to give some indication of the spread of the disease to the non-infected sinus.

TABLE VI

The control of turkey sinusitis by the potentiation of antibiotics used in treatment

Treatment	Feed conversion	Geometric rate of sinusitis		Geometric aerosaculitis	PPLO serology at end of Exper.*			
					Pen 1		Pen 2	
	Ave. both pens	One sinus	Both sinuses		Pos.	Neg	Pos.	Neg
Oxytetracycline 300 gms/ton reg. feed	7.03	60	37	25	5	0	2	3
Oxytetracycline 300 gms/ton low ca. feed	6.06	33	18	20	0	5	1	4
No drug - reg. feed	7.67	48	37	28	4	1	4	1
Non-infected control	5.85	0	0	0	1	4	—	—

* Positive serum - any agglutination in 1/25 dilution or higher.

SUMMARY AND CONCLUSIONS

The use of terephthalic acid (TPA) as well as a low calcium diet were studied to determine their ability to potentiate two tetracyclines, chlortetracycline and oxytetracycline. Both of these methods do increase the amount of antibiotic which is present in the serum. There appeared to be a two-fold increase in the clinical activity of both chlortetracycline and oxytetracycline against the disease, infectious synovitis. The mortality rate when 100 grams per ton of antibiotic was used with potentiation was similar to that when 200 grams were fed without potentiation. Therefore, in treating a disease which has antibiotic sensitivity similar to infectious synovitis, the use of a low calcium diet over a short treatment period will reduce by about one-half the feed level of antibiotic that is necessary to produce the beneficial clinical result. TPA as a potentiator, although not approved as a feed additive at this writing, appears to be very efficient. The use of low calcium (.6%) feeds as potentiators is justified for general field use.

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THE INFECTIVITY OF BLOOD AND TISSUES FROM CHICKENS WITH INFECTIOUS SYNOVITIS

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THE etiologic agent of infectious synovitis of chickens has been readily demonstrated in the synovial exudate, liver, kidney and spleen of naturally affected birds.[2,4,5,8] In experi-

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mentally infected birds, practically all tissues including the blood have been shown to contain the agent.[3] Bile has not yielded the agent and the intestinal contents usually have proved negative[3] although Wills[9] was successful in one attempt.

Cover and Benton[3] found that the agent was present in the blood by the 48th hour after either intravenous or intramuscular inoculation and persisted until the 10th day. Such periods were the minimum and maximum times tested.

The persistence of the agent beyond the acute state of the experimental disease has received only scant investigation. Wills[8] reported that liver was infectious on the 15th day after inoculation and in a subsequent report[9] found that joint exudate collected from birds inoculated three months previously failed to reproduce the disease.

The purpose of these experiments was to determine: (1) the time of appearance of the agent in the blood following inoculation, (2) the infective titer of blood at intervals after inoculation, and (3) the persistence of the agent in the blood and certain body tissues.

MATERIALS AND METHODS

The infectious synovitis agent used was originally isolated from a field case of the disease in 1954 and designated as strain 96. The agent as used in these experiments was maintained by consecutive chicken passage without intervening egg passage. Infective inocula for initiating the experimental disease were prepared from the livers and spleens collected from typically affected birds on the 7th to 10th days after inoculation. The organs were frozen in a commercial type deep freeze at –15 C immediately after harvest. Tissue suspensions were prepared by homogenizing the frozen organs in a Waring Blendor with sufficient sterile nutrient broth so as to obtain a 1:5 weight/volume dilution. The resulting material was centrifuged at 1000 rpm for five minutes in a size 1 International centrifuge. The supernatant was removed and placed in sterile screw cap tubes, cultured on bovine blood,

MacConkey's, and Sabouraud's agar to check for bacterial and fungal contaminants and placed in the freezer until used. This strain of the agent prepared in the manner described has routinely produced 90 to 100 percent mortality within 2 weeks when administered to 4 to 6 week old birds by the intravenous route.

Several breeds of chickens were employed as donors and for assay purposes with White Rocks and New Hampshires predominating. Vantress X Arbor Acre chickens were used as donors in some instances. All chickens were 4 to 6 weeks of age when inoculated except for certain of the donor birds as will be specified. Donor birds were inoculated by either the intravenous or intramuscular route.

Assay for the agent was accomplished by the intravenous inoculation of 0.5 ml of the test material. The agent was considered to be present in the test material if typical signs, lesions, or both, developed during a 3 or 4 week observation period. During this time, all birds were observed daily. All birds which remained alive at the end of the designated period were sacrificed and necropsied. In some instances, the test materials were also assayed in embryonated chicken eggs. The method used and criteria for the presence of the agent have been previously described.[3]

Blood for assay was obtained by cardiac or venous puncture. In most cases, a 2 percent solution of sodium citrate was used as an anticoagulant (0.1 ml per ml of blood) and the blood collected was mixed with an equal volume of nutrient broth. Sodium citrate in this concentration apparently has no deleterious effect on the synovitis agent.[1] In one experiment, the blood was allowed to clot and subsequently ground in a mortar with an equal volume of nutrient broth. Following centrifugation, as in the preparation of tissue suspensions, the supernatant was assayed. In those experiments in which infectivity titers were determined, serial ten-fold dilutions of the blood-broth mixture were made using sterile nutrient broth as a diluent. Each dilution was assayed in three birds and 50 percent chicken infective doses (CID_{50}) end points of titration were calculated by the method of Reed and Muench.[6]

EXPERIMENTS AND RESULTS

Onset of agent in blood. In order to determine the earliest demonstrable appearance of the agent in the blood after inoculation, the following experiment was conducted. Three susceptible chickens were inoculated by the intravenous route with 0.5 ml of an inoculum prepared from tissue of the third chicken passage. A like number were inoculated by the intramuscular route. Blood samples were obtained from each bird at intervals of 4, 8, 24, 28, 32 and 48 hours after inoculation and allowed to clot. Such samples were pooled on the basis of route of inoculation and interval of collection and assayed in three assay birds and five embryonated eggs.

The sample obtained on the 4th hour following intravenous inoculation failed to produce signs or lesions of the disease. Birds which received blood collected on the 8th hour did not die during the 3 week observation period, but two exhibited signs and had lesions at necropsy. Of the birds which received blood collected on the 24th hour, two died with lesions while the remaining bird exhibited signs and had lesions at necropsy. Birds receiving blood collected at intervals of 28, 32, and 48 hours after inoculation died during the observation period (tables 1 and 2).

Following intramuscular inoculation, samples obtained on the 4th through the 32nd hour failed to produce the disease while birds receiving blood collected on the 48th hour died during the observation period (tables 1 and 2). The results of the assay in embryonated chicken eggs were in agreement with the results in chickens.

TABLE I

Results of assay of blood collected at intervals following inoculation with the infectious synovitis agent

Route of administration	Post inoculation interval of collection and number of assay birds showing infection					
	4 hrs.	8 hrs.	24 hrs.	28 hrs.	32 hrs.	48 hrs.
Intravenous	0/3*	2/3	3/3	3/3	3/3	3/3
Intramuscular	0/3	0/3	0/3	0/3	0/3	0/3

* Number showing disease/number inoculated.

TABLE II

Occurrence of mortality in birds receiving blood collected at intervals following inoculation with the infectious synovitis agent

Route of administration and interval of collection	Mortality and day of occurrence
Intravenous - 8th hour	2/21st*
Intravenous - 24th hour	1/9th - 1/11th - 1/21st
Intravenous - 28th hour	2/10th - 1/11th
Intravenous - 32nd hour	1/9th - 1/10th - 1/12th
Intravenous - 48th hour	1/5th - 1/7th - 1/10th
Intramuscular - 48th hour	1/5th - 2/7th

* Number that died/day post inoculation of occurrence. Birds which showed lesions when sacrificed on the 21st day were considered to have died on that day.

Infective titer of blood at intervals after inoculation. Two experiments were conducted to determine the infective titer of the blood obtained at various intervals after inoculation. In the first, citrated blood samples were obtained on the 3rd, 6th and 10th days following inoculation by the intramuscular route with 0.5 ml of an inoculum prepared from tissue of the 3rd chicken passage. Two birds were sampled at each interval, the two samples pooled, and serial ten-fold dilutions prepared. Dilutions 10^{-1}, 10^{-3}, 10^{-4} and 10^{-5} were assayed for the agent. The sample obtained on the 3rd day after inoculation had a CID_{50} of $10^{-1.25}$ per 0.5 ml while samples obtained on the 6th and 10th days after inoculation had a CID_{50} of $10^{-4.60}$ per 0.5 ml (table 3).

In the second experiment, citrated blood samples were obtained on the 2nd, 4th, 6th, 8th, 9th, 10th, 11th, 12th, 13th and 14th days following inoculation from birds infected as in the previous experiment. Three birds were sampled at each interval, the samples pooled and serially diluted as above. In this trial, the undiluted blood broth mixture and dilution 10^{-1} thru 10^{-4} were assayed. The blood collected on the 2nd and 14th days was not infectious but those birds which served as donors on the 14th day had not exhibited signs of the disease prior to sampling. The CID_{50} per 0.5 ml of blood collected at the other intervals was as follows: $10^{-4.5}$ on the 4th, 6th, and 8th days; $10^{-4.0}$ on the 9th, $10^{-1.5}$ on the 10th, $10^{-4.5}$ on the 11th,

$10^{-3.0}$ on the 12th and $10^{-4.27}$ on the 13th (table 3). As the highest dilution used was 10^{-4}, the actual end point of titration on the 4th, 6th, 8th, 11th and 13th days was not determined but must be considered to be greater than the titer given.

TABLE III

Infective titer of blood collected at various intervals after intramuscular inoculation with the infectious synovitis agent

Days after inoculation	CID_{50} of blood*	
	Experiment 1	Experiment 2
2	—	0
3	1.25	—
4	—	4.5**
6	4.60	4.5**
8	—	4.5**
9	—	4.0
10	4.60	1.5
11	—	4.5**
12	—	3.0
13	—	4.27**
14	—	0

* Expressed as the reciprocal of the log per 0.5 ml of blood.
** Dilutions used were not sufficient to detect actual end points.

Persistence of agent in blood. As the results of the previous experiment indicated that the agent was still present in the blood on the 13th day after inoculation, an experiment was conducted to determine if it persisted beyond this time. A group of 40 chickens was inoculated by the intravenous route with an inoculum prepared from tissues of the 6th chicken passage and the disease allowed to run its course. On the 13th and 14th days following inoculation, citrated blood samples were obtained from each of three birds. On the 15th day only one bird remained alive for sampling. All samples were individually assayed for the agent.

Blood collected on the 13th, 14th and 15th days was infectious. Signs of the disease were present by the 5th day after injection and all birds died within a period of 9 to 13 days.

In an effort to obtain additional information on the persistence of the agent, chickens which had previously been

inoculated by the intravenous route and experienced the disease were sampled at irregular intervals after inoculation. Such chickens varied in age from 8 to 20 weeks when originally injected and were survivors of a group of 10 or more inoculated at that time. Two birds from a group inoculated when 16 weeks of age were sampled; the first, on the 13th and 15th days after inoculation and the second on the 15th, 19th and 21st days after inoculation. Two birds which received the agent when 8 weeks of age were sampled on the 52nd day after inoculation. Five birds which had received the agent when between 14 and 20 weeks of age were used. Two were sampled 64 days, one, 78 days and two, 112 days, after inoculation.

Blood collected on the 13th and 15th day after inoculation from one bird inoculated at 16 weeks of age was infectious while blood obtained on the 15th, 19th and 21st day after inoculation from another bird of the same group was not infectious. Blood collected on the 52nd day after inoculation from birds infected at 8 weeks and on the 64th, 78th and 112th day after inoculation from birds infected when between 14 and 20 weeks of age was not infectious.

Persistence of agent in tissue of chronically affected birds. In order to determine if the agent localizes and remains viable in certain tissues, the liver, spleen, kidney, ovary or testis and joint exudate were obtained from each of three chronically affected birds. The first of these was a female and had received the agent 128 days previously when 16 weeks of age; the other two birds were males and one had received the agent 114 days previously when 18 weeks of age, while the other had received the agent 100 days previously when 20 weeks of age. All birds exhibited chronic synovial lesions, i.e. sternal bursitis, enlarged joints and clear to yellowish or orange exudate in the hock joint and along the tendons. Internal organs appeared normal on gross examination. A portion of each organ was selected and prepared in the same manner as that used for tissue suspensions previously described. Joint exudate was also diluted 1:5 with sterile

nutrient broth. All such materials were assayed in five birds for the presence of the agent. No evidence of the disease was observed in the assay birds over a 4 week observation period.

DISCUSSION

The results of the experiment on the onset of the infectious synovitis agent in the blood indicates that an infectious level is reached more rapidly following intravenous inoculation than intramuscular inoculation. The time that the agent is first present in the blood stream at an infectious level probably varies with other routes of inoculation.

The pattern of mortality in the assay birds suggests that there is an increase in the infectivity of the blood during the first 48 hours after intravenous inoculation. The results obtained when the 48 hour sample from intramuscularly inoculated birds was assayed would indicate that a rapid increase occurs somewhere between 32 and 48 hours after inoculation.

The experiments concerned with the infective titer of the blood at intervals after inoculation indicate that such a titer probably increases until 72 to 96 hours after inoculation. Although some inconsistencies occurred, the titer remained relatively constant through the 13th day, the longest period tested. The failure to obtain infection from samples obtained on the second day of the second experiment conceivably may have been due to selection of birds which subsequently failed to develop the disease. Similarly, the drop in the infectivity titer noted on the 10th day may have been an accident, especially in view of the titer obtained on subsequent days. Whether the use of higher dilutions in the second trial would have established a peak and subsequent decline of titer is, of course, open to question.

The agent was still present in the blood of birds previously inoculated by the intravenous route on the 15th day after inoculation. One exception to this was observed in samples obtained from one bird previously inoculated when 16 weeks of age. The possibility that the age at which a bird is infected influences the persistence of the agent in the blood must be considered.

Failure of blood collected on the 52nd day after inoculation and later or of tissues from chronically affected birds to infect susceptible birds may indicate complete absence or low infectiousness of the agent. Snoeyenbos[7] has recently reported the isolation of the agent from chicken embryos only after four consecutive passages in embryonated eggs. At any rate, the agent does not appear to remain at infectious levels for an extended period of time.

SUMMARY

The infectious synovitis agent was studied with regards to its presence and persistence in the blood of experimentally inoculated chickens and its persistence in certain tissues of chronically affected chickens. The results indicate:

1. That the agent is present in infectious levels in the blood by the 8th hour after intravenous inoculation but not by the 4th. Following intramuscular inoculation, an infectious level is attained at the 48th hour but is not present at the 32nd hour.

2. The infective titer of blood from chickens inoculated by the previous routes appears to increase until 72 to 96 hours post inoculation after which it becomes relatively constant through the 13th day.

3. The agent persists in the blood until at least the 15th day after inoculation.

4. The agent is not present in blood obtained 52 days after inoculation or later or from liver, spleen, kidney, ovary, testis or joint exudate obtained from chronically affected birds.

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COLIFORM PERITONITIS OF CHICKENS*

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PERITONITIS characterized by a fibrinous exudate that often has the appearance of a coagulated egg yolk, is a common cause of mortality on laying flocks. This condition was frequently observed during the routine examination of dead and sick chickens from the Virginia Polytechnic Institute Poultry Department. It was noted that in all cases, peritonitis occurred only in ovulating females. Daily trap nests records were available for 31 of these and the time since the last egg was layed is recorded in table 1. Cole and Hutt[1] report the existence of hens which ovulate but do not lay even though they have the physical appearance of good layers. They also suggest that absorption of yolk in the peritoneal cavity is rapid. Cultures of coliform bacteria were isolated from the peritoneal cavities of the few live birds submitted. These isolates proved to be of the same serological types as those associated with periocarditis.[2]

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MATERIALS AND METHODS

Two identical experiments were conducted to determine the relationship of pathogenic strains of *Escherichia coli* and egg yolk in the etiology of peritonitis. Eighty-four adult female chickens known to be laying were obtained from the V. P. I. Poultry Department for these experiments. One half of the chickens were inoculated with 25 ml of sterile egg yolk into the peritoneal cavity. This was accomplished by inserting a 13 gauge needle horizontally just ventral to the vent and injecting the yolk with a 25 ml syringe. In order to minimize the chance of carrying coliform bacteria from the skin into the body cavity, the skin at the site of injection was washed and painted with iodine and a separate needle was used for each chicken. One-third of the chickens were inoculated into the peritoneal cavity with 0.1 ml of a 10^{-2} dilution of a 24 hour broth of type 2-1-? *E. coli*, containing approximately 10^6 bacteria. Another third were given 0.2 ml. of the same culture, undiluted into the vagina within ½ hour of the yolk injection. The incidence of peritonitis and mortality were recorded and are summarized in table 2.

RESULTS

The results indicate that egg yolk increased the pathogenicity of *E. coli*. Administration of *E. coli* into the vagina when yolk was given intraperitoneally resulted in peritonitis in six of 14 birds. Of the 12 birds which died in these experiments, three died during the 1st day after the inoculation, seven died during the 2nd day and two during the 3rd day. Experimentally produced lesions were as severe as those of naturally infected birds.

TABLE I

Time of last recorded egg of chickens with peritonitis

Days before death that last egg was recorded	Number
No eggs recorded	7
1-10	9
11-25	5
26-50	6
51-100	3
101-150	1

TABLE II

Incidence of peritonitis and mortality following inoculation of mature chickens with sterile yolk and *Escherichia coli*

	Coli IP		Coli Vagina		No Coli	
	D	L	D	L	D	L
Yolk IP	8/14*	10/14	4/14	6/14	0/14	0/14
No Yolk	0/14	2/14	0/14	0/14	0/14	0/14

D = deaths L = lesions,
* numerator = number dead or with lesions, denominator = number in the group.

DISCUSSION

From the results of these experiments and the findings of Cole and Hutt the following sequence of events can be postulated for the natural development of peritonitis in layers. Pathogenic coliforms may live for long periods of time in the intestinal tract of normal chickens without causing any visible disease.[3] A small amount of fecal matter could deposit a large number of bacteria into the vagina from which they could gain access to the oviduct. Ordinarily these are controlled by the birds defenses and no disease results. At times ovulation occurs and the infundibulum does not pick up the ovum. The vitelline membrane ruptures and the peritoneal cavity is contaminated with yolk. Ordinarily the yolk is rapidly absorbed and no disease results. If coliforms enter through the oviduct while yolk is in the body cavity peritonitis can result.

SUMMARY

Peritonitis was produced in chickens by inoculating sterile egg yolk intraperitoneally and at the same time a pathogenic strain of *Escherichia coli* intraperitoneally or into the vagina. It was suggested that peritonitis naturally occurs when the peritoneal cavity is contaminated with yolk and *E. coli* enters through the vagina.

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TYPING OF *SALMONELLA PULLORUM*. THE INFLUENCE OF METHODS ON RESULTS*

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IN 1946 Edwards and Bruner[1] studied form variation in *Salmonella pullorum* and its relation to the so-called *X strains*. They concluded that the antigenic formula of *S. pullorum* is $9,12_1,(12_2),12_3$. The 12_2 factor is variable and forms that contain a large amount or a negligible amount of 12_2 can be isolated from the same culture. These forms may stablize in standard or variant (X) strains. The standard strain contains a large amount of 12_3 and a very small amount of 12_2 whereas the content of these two antigens in the variant strain is reversed. Edwards and Bruner[2] used absorbed serums to distinguish between the standard and variant strains. *Salmonella paratyphi A* var. *durazzo* serum $(2,12_1,12_3)$ ab-

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** Technical assistance by Esther E. Moffat.

sorbed by *Salmonella reading* ($4,12_1,12_2$) was used to select the forms that possessed a large amount of 12_3 and *S. reading* absorbed by Durazzo for those predominately 12_2.

Similar serums have been used in this laboratory for the routine typing of *S. pullorum* cultures sent in by the service laboratories of the New York State Veterinary College. While conducting this typing it was observed that isolates which behave as standard forms when the whole culture is tested are not necessarily limited to the 12_3 form. The examination of a sufficient number of single colonies from these isolates frequently reveals a second form that carries both 12_2 and 12_3 components. This one is referred to as the intermediate form. It possesses all the antigens in the formula set forth by Edwards and Bruner.[1]

Because the testing of whole *S. pullorum* cultures did not produce an accurate picture with regard to the forms present, it was decided to investigate the typing procedure. Furthermore it was resolved to conduct the tests using homologous absorbed serums rather than the heterologous ones employed in previous studies.

MATERIALS AND METHODS

Highly immunogenic standard and variant strains of *S. pullorum* were selected by testing single colonies. These were used according to the following schedule in immunizing rabbits by means of intravenous inoculation.

1. 0.2 ml—1st day
2. 0.5 ml—3rd day
3. 1.0 ml—5th day
4. 2.0 ml— 9th day
5. 5.0 ml—13th day
6. Bleed—17th day

Single factor 12_2 and 12_3 antiserums were prepared by conducting cross absorptions. Excellent serums could be obtained by making double absorptions of 1 ml of antiserum with the growth harvested from 12 tryptose agar plates. The absorbed serums were used in titers of about 1:6 in plate testing the *S. pullorum* cultures.

RESULTS

When 147 isolates of *S. pullorum* derived from outbreaks

of disease in fowl were tested as whole cultures with single factor 12_2 and 12_3 serums they were identified as 109 standard, 21 intermediate, and 17 variant forms.

By plating the 109 standard cultures and testing 10 single colonies of each, it was demonstrated that 76 showed only standard forms while 33 produced at least one or more intermediate colonies. Ten of the 76 cultures that appeared to be standard on the basis of the identity of 10 single colonies were then plated and 100 colonies of each were tested. One culture of the 10 produced 100 standard colonies. The nine remaining all showed intermediate forms which were revealed on the 60th, 52nd, 47th, 31st, 27th, 26th, 21st, and 17th tests respectively.

When growth from single standard colonies was plated serially, the progeny usually were standard in form but intermediate forms also appeared. The ratio of the occurrence of the intermediates was not determined at this time. Serial plating of growth from single intermediate colonies resulted in progeny both standard and intermediate in form in the ratio of about 6:4.

Plating of the 21 cultures that as whole cultures had reacted both with the 12_2 and 12_3 serums revealed 15 intermediate strains and 6 mixed. The six mixed cultures showed the following standard:variant ratios based on the indentities of 10 single colonies in each case; 9:1, 8:2, 5:5, 4:6, 4:6, 3:7. The 15 intermediate cultures produced only standard and intermediate forms.

Plating of the 17 variant cultures and examination of 10 single colonies of each resulted only in variant forms. In three instances the testing of 100 single colonies revealed no change in form.

DISCUSSION

It appears from these studies that many of the *S. pullorum* isolates made by the service laboratories of the New York State Veterinary College are a mixture of intermediate and standard forms, the standard predominating. In fact our study poses the question whether completely standard cultures are ever isolated?

It also appears that the variant form of *S. pullorum* is much more stable than the standard form. In our hands a single intermediate colony readily produced standards as well as intermediate, but not variant forms. In turn the resulting standard colonies produced other standard and also intermediate forms. Variant forms treated in the same way remained stable.

CONCLUSIONS

Single factor serums (12_2 and 12_3) were used in the rapid plate method to examine 147 isolates of *Salmonella pullorum*. When tested as whole cultures these isolates were identified as 109 standard, 21 intermediate, and 17 variant forms. By plating the 147 isolates and testing single colonies 67 standard, 57 intermediate, 17 variant, and 6 mixed forms were revealed.

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AN INFECTIOUS AGENT PRODUCING HEPATITIS IN TURKEYS

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DURING June of 1958, a group of turkey poults exhibiting lesions which appeared to be different from those previously observed by the authors were submitted to the diagnostic laboratory. At the time of examination, a mortality of 12 to 15 had occurred during the previous 72 hours in a group of 800 17-day-old birds. Slight depression in activity of the flock was reported. The flock returned to normal appearance during the following 3 to 4 days and only a few additional birds died. The poults had been bred and hatched on the premises. A variety of other age groups on the farm appeared to be unaffected both then and later.

The eight specimens submitted (three alive and five dead) exhibited normal growth and fleshing. The gastrointestinal tracts were full of feed. Dehydration was not apparent. Lesions were confined to the liver and pancreas. The liver was enlarged and studded throughout with small light colored foci. Vascular injection or hemorrhage was prominent and partially obscured the degenerative changes. The pancreas contained rather large degenerative appearing foci. Lesions were remarkedly striking and uniform in seven of the eight specimens.

Consistently negative results were secured in attempts to

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cultivate an agent on artificial media at 37 C. Both infected ground liver tissue and yolk were used. The following media and methods were employed: 10 percent chicken blood in an agar infusion base incubated under atmospheric conditions as well as 10 percent added CO_2 in a moist chamber, chicken infusion agar and broth, thioglycollate medium, brain heart infusion broth and agar with 10 percent horse serum, and Grumbles' phenol red carbohydrate broth.[1]

A ground liver suspension from several specimens was injected into the yolk sac of 7-day-old chicken embryos. All embryos died between the 4th and 11th day postinjection and were demonstrated to be free of bacteria. Day-old chicks injected intravenously with similar liver material failed to exhibit clinical abnormality or gross lesions at 3, 7, 10 and 14 days postinjection. Similarly injected poults developed light-colored, apparently degenerative, foci in the liver demonstrated at 7 and 10 days postinjection.

A group of eight 21-day-old mice were injected with a suspension of infected liver tissue. Four of the mice were injected intracerebrally with 0.05 ml; the others were injected with 0.1 ml intraperitoneally. Both groups remained clinically normal and were grossly normal at necropsy 14 days later.

At this point it appeared likely that an uncatalogued infectious agent had been isolated which warranted further investigation. A series of trials were performed to gain some insight of the agent and the disease produced.

Shortly after the disorder was found to be a distinct entity, it was recognized that a similar or identical disorder had repeatedly been observed since 1955 at another diagnostic laboratory* in the state. One field case was subsequently observed and livers were collected and forwarded to the authors; an apparently identical agent was isolated.**

Formalinized tissues were secured from North Carolina*** from a field case suggestive of the disorder; changes

* Branch Poultry Diagnostic Laboratory, Waltham Field Station. The assistance of Dr. G. P. Faddoul in providing histories and tissues is gratefully acknowledged.

** Isolations from two additional cases, one originating from New Hampshire, have been made since preparation of this manuscript.

*** Furnished by Dr. W. C. Clements, Monroe, North Carolina.

highly suggestive of the disease were found in these tissues. It was also learned that a similar or identical disorder had been observed in Minnesota for a considerable number of years.[2]

MATERIALS AND METHODS

Chicks and fertile eggs used in these studies were produced by a flock of Rhode Island Reds demonstrated to be free of pathogenic PPLO infection. Turkey poults were secured from flocks of either Broad Breasted Bronze or Broad Breasted Whites which had not, to our knowledge, ever shown evidence of the infection under study.

The original isolate designated #356 was used throughout the series of trials reported. All embryo passages of the agent were carried out by injection of 0.2 ml of undiluted yolk into the yolk sac of 7-day-old embryos unless otherwise indicated. Isolations from experimentally infected birds were done by grinding the tissue in approximately four parts of nutrient broth in a Ten Broeck grinder and injecting 0.2 ml of the suspension into the yolk sac of six or more 7-day-old embryos. Heparinized blood and bile were used undiluted for isolation; feces were diluted approximately 1 to 3 in nutrient broth, centrifuged to clear the suspension, and the supernatant filtered through a Seitz EK filter before embryo injection. Injected embryos were candled daily. Mortality during the first 48 hours was discounted as being nonspecific. Embryos alive on the 20th day of incubation were chilled and examined. Yolk fluid of dead embryos was routinely cultured in thioglycollate broth to insure bacterial sterility. Harvested fluids or tissues were held at –20 C.

Trial birds were maintained in wire floored batteries and fed commercial rations. Yolk propagated agent was used undiluted to initiate infection. In the trial to test susceptibility of the gastrointestinal tract, the agent was administered in double walled gelatin capsules using the basic technique described by Winterfield *et al.*[4]

RESULTS

Behavior in embryos. Passage in the yolk sac of chicken

embryos has produced rather consistent results. A typical mortality pattern is shown in table II under embryo assay of drug sensitivity. Mortality occurred chiefly between the 4th and 11th day postinjection (PI). Occasional mortality occurred on the 3rd day PI as well as after the 11th day. Mortality was usually 100 percent except in instances of apparent low titered inoculum; in such instances, one or occasionally two passages in embryos resulted in the usual complete mortality.

Stunting and vascular injection or hemorrhage of the integument was quite consistently observed. Embryos which died after the 11th day PI showed marked stunting, but less consistent and less prominent integumentary changes than did those which died earlier. Gross liver lesions consisting of necrosis and hemorrhage were also frequently observed after the 11th day.

Injection of yolk-propagated agent on the chorio-allantoic membrane of 10-day-old embryos failed to produce lesions at the site of injection, but produced occasional embryonic mortality late in the incubation period. Injection into the amnio-allantoic (aa) sac at the 10th day of incubation produced occasional mortality and embryonic lesions. Three serial passages from aa flund to the aa sac were accomplished.

The agent was readily propagated in the yolk sac of turkey embryos injected on the 7th day of incubation. Embryonic changes were similar to those produced in chicken embryos.

Titration of four separate yolk harvests of the agent, calculated according to the method of Reed and Muench,[3] demonstrated embryo lethal dose$_{50}$ (ELD_{50}) titers of from $10^{-2.0}$ to $10^{-4.2}$.

Experimental exposure of turkeys and chicks. Trial I which is summarized on table 1 was designed to test infectivity of the agent for chicks, and to indicate the response of poults to intraperitoneal injection of the agent. Non-injected battery mates served as contact controls. In group A, the agent had previously been passed three times in embryos and three consecutive times through turkey poults following initial isolation; in the B groups the agent had been passed six times

in embryos. At 1 through 7 days PI and on the 10th, 12th, and 14th day, two randomly selected poults and two chicks from each injected group were sacrificed. One poult from each contact group was also sacrificed and processed. Liver tissue was collected for egg embryo injection. Gross lesions were recorded and the following tissues were collected in 10 percent formalin for histological examination: liver, spleen, brain, kidney, lung, proventriculus, gizzard, pancreas, duodenum, and femoral bone marrow.

TABLE I

Response of 2-day-old turkeys and chickens to intraperitoneal injection of the turkey hepatitis agent

	Days P. I.	Turkeys Lesions			Chickens	
		Injected	Contact	Isolation	Lesions	Isolation
G	1	0/2	0/1	—	0/2	—
R	2	0/2	0/1	+	0/2	+
O	3	0/2	0/1	+	0/2	—
U	4	1/2	0/1	+	0/2	—
P	5	0/2	0/1	+	0/2	—
	6	2/2	1/1	+	0/2	—
A	7	2/2	1/1	+	0/2	—
	10	1/2	0/1	+	0/2	—
	12	1/2	1/1	+	0/2	—
	14	2/2	1/1	+	0/2	—
G	1	0/2	0/1	+	0/2	—
R	2	0/2	0/1	+	0/2	—
O	3	0/2	0/1	+	0/2	—
U	4	0/2	0/1	+	0/2	—
P	5	0/2	1/1	+	0/2	—
	6	2/2	0/1	+	0/2	—
B	7	1/2	0/1	+	0/2	—
	10	2/2	1/1	+	0/2	—
	12	2/2	1/1	+	0/2	—
	14	1/2	1/1	+	0/2	—

All injected birds received 0.2 ml IP of infected yolk. The A group received the agent which had previously been passed 2 times in chicken embryos and 3 times in poults. The B group received the agent which had previously been passed 6 times in chicken embryos.

The two methods of propagating the agent appeared to have no effect on the infectivity or virulence of the agent. Lesions were found in the poults as early as the 4th day following injection and as early as the 5th day in the contact birds; gross lesions were quite consistently found thereafter to the

termination of the trial at the 14th day PI. No gross changes were detected in the chicks.

The agent was re-isolated on the first embryo passage from each poult liver pool on each attempt during the 2nd to 14th day PI. One of the liver pools at 24 hours PI yielded the agent, but it apparently was present in a low titer as typical embryonic mortality did not occur until the second passage. Only one isolation, on the 2nd day, was accomplished from chick livers. This too appeared to be in low concentration as typical embryonic mortality was not secured until the 3rd embryo passage.

Trial II was for the purpose of extending the observation period of trial I in relation to duration and progress of lesions, and the period during which the agent could be isolated from the livers of infected turkeys. One-week-old poults were injected intraperitoneally with 0.2 ml of yolk from the sixth embryo passage of the agent. A group of uninjected poults were maintained in separate batteries in the same room. Two injected and two uninjected birds were necropsied at 24 hour intervals from the 3rd through the 9th day PI. Two injected birds were necropsied and livers saved for isolation at 4-day intervals between the 14th and 36th day PI. Tissues were also formalinized for sectioning as in trial I.

Gross lesions were first observed in the injected group at the 5th day PI. Lesions were found in the room contacts 96 hours later. Gross lesions were observed at the 14th and 20th day PI but not at 24 days or later. The agent was recovered from all groups in which gross lesions were observed.

In trial series III, groups of eight poults at 2, 4, 6, 8 and 10 weeks of age were injected intravenously with 0.2 ml of yolk propagated agent. Two randomly selected birds from each group were sacrificed at the 3rd, 5th, 6th and 10th day PI. Liver tissue from each group was saved for isolation of the agent. A single 12-month-old turkey hen was also injected.

Gross hepatic and occasional pancreatic lesions were observed in all age groups. Lesions were first detected on either the 5th or 6th day PI in each group. The agent was recovered from livers of each group. Hepatic lesions were observed and the agent recovered from the turkey hen when

examined seven days after injection. It did not appear that there were significant differences in severity of lesions between the different age groups.

In trial IV, three groups of six 5-week-old turkeys were maintained in separate isolation areas and exposed by various routes to 0.2 ml of infected yolk. Group I was exposed by instillation into the trachea, group 2 by administration of double walled capsules into the crop, and group 3, by addition of the agent to the drinking water. Two birds from each group were necropsied on the 4th, 6th and 8th day PI. Lesions were found in the livers and the agent isolated from each group on the 6th and 8th day PI.

Distribution of the agent in affected turkeys. The agent was isolated in embryos from heart blood, liver, kidneys, and feces collected on the 4th day PI from poults infected intravenously at 21 days of age. The agent was not isolated from bile or heart at this time. Further trials are being conducted to define the period that these tissues and materials contain the agent.

Histopathology. Microscopic examination of tissue sections from turkeys indicated that the only pronounced lesions occurred in the liver and pancreas. Fatty metamorphosis was seen in the liver during the first 2 days PI. On the third day, mononuclear foci and focal parenchymal necrosis appeared in many areas. The frequency and severity of these changes were most pronounced about the 7th day. Necrotic foci were not seen in the liver after the 10th day PI, but mononuclear foci in the pancreas were most numerous about the 14th day. Lesions were not found in these organs from birds sacrificed at the 24th day PI and thereafter. Lesions in experimental turkeys were similar but not nearly as pronounced as those seen in field cases. Moreover, hemorrhage in the liver and focal parenchymal necrosis of the pancreas were common findings in turkeys which died from field infection. Tissues from experimentally injected chicks had no significant lesions.

Drug sensitivity. The agent was tested in ovo for sensitivity to various drugs. The following were tested: tetra-

cycline, chlortetracycline, oxytetracycline, dihydrostreptomycin sulfate, polymixin B sulfate, penicillin G, bacitracin, and micronized furazolidone. The drugs were dissolved or suspended in normal saline to produce a concentration of 10 mg per ml. Each drug preparation was then mixed singly with an equal volume of yolk-propagated agent containing 1000 ELD_{50} per ml and incubated at room temperature for 30 minutes prior to injection into the yolk sac of 7-day-old embryos. Each injection of 0.2 ml contained 1 mg of the drug tested and 100 ELD_{50} of the agent.

The embryo mortality summarized on table II demonstrates an unchanged pattern following treatment with the drugs tested.

Effect of filtration and centrifugation. Filtration trials were performed with affected livers prepared as described for isolation in embryos. The tissue suspension was centrifuged to remove most of the cellular elements and passed through a Boerner type Seitz EK filter. The filtrate was then used for passage through the desired size of Millipore filter. The final filtrate was injected in 0.2 ml quantities into the yolk sac of 7-day-old embryos to determine the presence of the agent. In one trial this procedure was modified by grinding in four volumes of distilled water and freezing and thawing four times prior to centrifugation; after centrifugation the supernatant was passed successively through Seitz EK and 0.45, 0.30, and 0.10 micron Millipore filters.

The agent readily passed through Seitz EK filters and 0.45 micron Millipore discs. It also consistently passed through 0.3 micron discs but with a considerable apparent loss of titer indicated by partial embryonic mortality on first passages. In six attempts, the agent was not demonstrated to pass through a 0.1 micron Millipore filter.

In the centrifugal sedimentation trials, infected turkey livers were ground as described for embryo isolation. The tissue suspension was then centrifuged at sufficient force to sediment most cellular material and the supernatant filtered through a Seitz EK filter. The filtrate was then centrifuged for one hour in a refrigerated angle centrifuge, decanted and

TABLE II

Drug sensitivity of the turkey hepatitis agent tested in 7-day-old embryos

Drug tested	*Amount of drug Injected per embryo		No.	Days postinjection and embryo death													No. deaths
	Weight mg.	Units (calculated)	Eggs	1	2	3	4	5	6	7	8	9	10	11	12	13	No. injected**
uninjected controls			8														0/8
injected controls			8	2			2		2		1	1					6/6
Tetracycline	1.0	1,000	8				4	1	1		1	1					8/8
Chlortetracycline	1.0	1,000	8				3	2	1	1			1				8/8
Oxytetracycline	1.0	1,000	8				2	1		2	2		1				8/8
Dihydrostreptomycin	1.0	799	8	1		1	2	2	1		1						7/7
Polymixin B	1.0	10,000	8				3	1	1	1	1					1	8/8
Penicillin G	1.0	1,595	8			1	2		1	1	1	1	1				8/8
Bacitracin	1.0	71	8	1			3	2	1		1						7/7
Furazolidone (Micronized)	1.0		8				2	2	2	1							7/8

* Amounts tested were nontoxic for embryos in parallel tests
** First 48 hour mortality discounted as nonspecific
100 ELD_{50} injected per egg of yolk passage isolate 356

the supernatant again centrifuged for one hour. Each supernatant was injected in 0.2 ml quantities into the yolk sac of eight 7-day-old embryos. The agent was isolated from the supernatant after one and two hours centrifugation at 25,000 times gravity.

Effect of temperature. Trials were conducted to ascertain the viability of the agent under several different temperatures. Undiluted yolk was used as the source of the agent. Groups of eight 7-day-old embryos, injected via the yolk sac, were used as an index of viablity of the agent. Actual titration of the agent was not attempted.

The agent was demonstrated to remain viable after 365 days, the longest period tested, if held in screw cap vials at –20 C. Viability was likewise demonstrated after 14 but not 16 hours in a water bath at 56 C. Viability was demonstrated at 5 hours, the longest period tested, at 60 C.

DISCUSSION

Some of the characteristics of a contagious disease of turkeys, apparently not previously described, are presented. Available field information indicates that the disease, primarily a hepatitis, may produce appreciable mortality in young turkeys. Two confirmed cases of the infection resulted in a mortality of approximately 2 percent in one case and 20 percent in the other. In the latter case, mortality extended over a two week period.

Available diagnostic records of cases dating from 1955 strongly suggest that the disease was responsible for at least 11 field infections in Massachusetts prior to initial isolation of the causal agent. In these presumptive cases, poult ages ranged from 9 to 35 days. Mortality at the time of submitting specimens varied from one to six percent, and was considerably higher in several cases in which intercurrent infections were recognized. In three instances, mortality occurred on the same farm after intervals of a year or more. Most histories indicated little or no apparent morbidity even during the period of highest mortality. In most cases, no abnormality was reported in other age groups in the farm; this was true of confirmed as well as presumptive cases.

Available information indicates that the disease has also been responsible for mortality in turkeys in both North Carolina and Minnesota. If this is true, it may be anticipated that the agent is also present in other areas.

Under experimental conditions, infection of the turkey was produced with ease, and direct or indirect transmission readily occurred. Neither mortality nor clinical signs have been produced in several hundred experimentally infected turkeys of a variety of ages. Although infection and gross lesions were consistently produced, the lesions were relatively minor in extent as compared to those observed in field mortality. It appears that under field conditions the infection may commonly occur in flocks without the development of clinical signs.

The chick does not appear to be susceptible to the agent. No lesions, gross or microscopic, were found following injection. In only one instance was the agent isolated from tissues of an injected chick. This occurred 48 hours PI and probably represented residual agent as a consequence of injection.

It appears likely that the causal agent is a virus. This view is supported by filtration studies indicating a particle size of 300 $m\mu$ or less, failure to be sedimented after 2 hours at 25,000 times gravity, failure to grow on a broad range of standard bacteriological media, and lack of susceptibility to a broad range of antibiotics.

SUMMARY

An acute infectious, apparently highly contagious, hepatitis has been recognized in turkey poults. The agent, probably a virus, is readily cultivated in the yolk sac of chicken embryos; it passes through an 0.3 micron Millipore filter and appears unaffected by antibiotics. A focal degenerative hepatitis occurs in affected poults which may also develop hepatic congestion or hemorrhage and degenerative changes in the pancreas. Chicks have proved refractory to the agent. Available evidence indicates that the disease is present in at least several areas of the United States.

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VIRUS HEPATITIS IN TURKEYS

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ON March 20, 1958 a group of young turkey poults was sent to the post mortem laboratory at the Ontario Veterinary College with a history of reduced hatchability and a higher than usual mortality, starting on the 9th and 10th day of life. The group was part of a weekly hatch of 5,700 poults. The eggs were imported from California and were hatched on the turkey farm. The poults were fed two pounds of furazolidone (NF 180) per ton of feed until they were 2 to 3 weeks of age.

On post mortem examination, the livers were found to be enlarged and had grayish areas of necrosis ranging in size from less than one millimeter to four or five millimeters in

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diameter. Congestion and pin-point hemorrhages were also evident. Microscopic examination of sections of the livers showed small, necrotic foci with granulocytic infiltration and hemorrhages. Samples of heart and liver gave negative results on blood agar and MacConkey's agar. Liver was ground in tryptose phosphate broth containing 1,000 units of penicillin and 2 mgs streptomycin per ml and the suspension was centrifuged. The supernatant was inoculated into the yolk sac of six 8-day embryonated eggs. There was one death on the 7th day, one on the 10th day, two on the 11th day and one on the 12th day. Subsequent passages in embryonated eggs usually resulted in 100 percent mortality between the 4th and 8th day after inoculation. (table 1). Yolk, harvested from dead embryonated eggs, and plated on blood agar and PPLO agar and incubated aerobically, anaerobically or under 10 percent CO_2 was negative for bacterial growth.

A diagnosis of infectious hepatitis was made and the following experiments were designed in an attempt to determine the nature of the causative agent.

EXPERIMENTAL

Twenty day-old poults were obtained from a source thought to be free of the agent being investigated. Ten of these were maintained as controls. Yolk, harvested from embryos that had died following injection of the agent, was diluted 1:1 with tryptose phosphate broth containing 1,000 units of penicillin and 2 mg of streptomycin per ml. The remaining ten birds were each given 0.4 ml of the diluted yolk. The unabsorbed yolk sac was used as the site of inoculation.

Seven days after inoculation, the poults were killed and examined. Macroscopically the livers showed small grayish lesions similar to those of the original field case, although the lesions were fewer in number (figure 1). A microscopical examination showed lesions varying from small necrotic foci surrounded and infiltrated by leukocytes, to solid areas of reticuloendothelial cells where no necrotic material remained (figures 2, 3, 4).

A suspension made from the affected livers caused deaths in embryonated eggs. Portions of the livers were also used

TABLE I

Death pattern of chicken embryos

Key	Inoculum	Age of embryos (days)	Embryo mortality—days after inoculation Yolk sac route													No. deaths / No. inoc.
			1	2	3	4	5	6	7	8	9	10	11	12	13	
A	Original liver suspension	8		1					1			1	2	1		6/7
B	Yolk from A	6			1	1		1	2	1						6/8
C	Yolk from B	6		1		2		1	1						1	6/6
D	Yolk from C	6				1		3	1	1						6/6
E	Yolk from D	6				2		1		2	1					6/6
Liver suspension from infected poults																
7 days post inoc.		7					3	3	2							8/8
4 days post inoc.		6					1	1	2	2	2					8/8

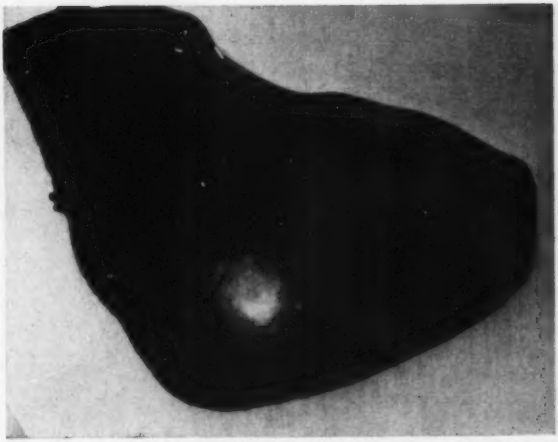

Fig. 1. Liver of an experimentally infected turkey poult necropsied seven days after inoculation. The hematocyst is an artifact.

to make a suspension for inoculation into more day-old poults; seven passages were made (table 2). In other experiments using the intravenous route of inoculation, it was found that lesions could be produced in day-old poults within 4 days, and that poults were susceptible up to at least 35 days of age.

Infective yolk inoculated into the allantoic cavity of 10-day-old embryonated eggs gave negative results. Five trials with day-old chicks, using the unabsorbed yolk sac as the inoculation site, were unsuccessful. Despite these negative results, the effect of the agent on chicks should be investigated more fully.

Filtration. Livers from infected poults were ground with sterile alundum in grinding tubes, and a suspension was made in tryptose phosphate broth. This suspension was centrifuged at 2,000 r.p.m. for 20 minutes and the supernatant passed through a Seitz EK filter pad. Portions of this filtrate were then passed through 0.45, 0.3 and 0.1 micron membrane Millipore filters. The integrity of the filters was ascertained by the use of a broth culture of *Serratia marcescens.* Each

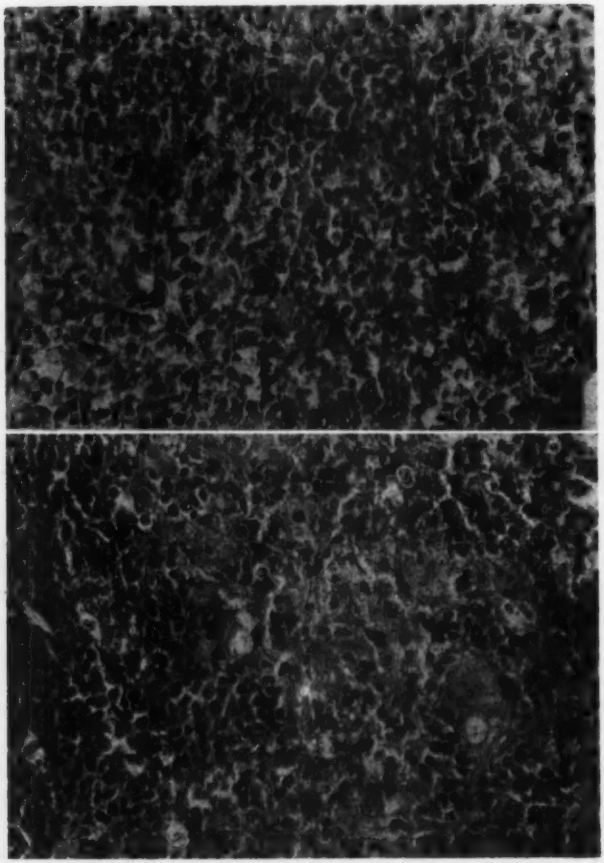

Fig. 2. Focal necrosis and cellular infiltration. The infiltrating cells are mainly lymphocytes but a few granulocytes are also present. Hematoxylin and eosin. 450×

Fig. 3. Massive inflammatory cell infiltration and proliferation replacing liver parenchyma. Only a few liver cells remain. Granulocytes are absent. Hematoxylin and eosin. 450×

filtrate was used to inoculate 10-day-old poults intravenously using 0.4 ml in each bird. Ten other birds were inoculated in a like manner with the centrifuged material and ten birds

served as controls. Portions of each filtrate were also used to inoculate eight 6-day-old embryonated eggs via the yolk sac.

The poults inoculated with the centrifuged material and the filtrate from the Seitz EK and the 0.45 micron Millipore filter each showed several liver lesions. In each group which had received the filtrates of the 0.3 and 0.1 micron Millipore filter there was only one poult with one liver lesion (table 3). Each of the above filtrates when inoculated into embryonated eggs caused deaths (table 3).

TABLE II

Reproduction of the hepatitis in poults

Age of poults at time of inoculation (Days)	No. affected / No. inoc.	Recovery of the agent in embryos No. of days after inoculation		
		4	8	11
1	7/14	ND′	+	ND
1	10/14	ND′	+	ND
1	16/20	ND	—	+
1	6/10	+	ND	ND
4	7/10	+	ND	ND
4	9/9	+	ND	ND
32	2/7	ND	ND	ND

′ Not done.

TABLE III

Filtration experiments

	Lesions in poults		Mortality in embryos	
	No. affected / No. inoculated		No. deaths / No. inoculated	
Filter	Exp. 1	Exp 2	Exp. 1	Exp. 2
Seitz EK	8/12	ND′	6/8	ND′
450 mμ″	6/12	17/26	6/8	4/8
300 mμ	1/8	7/20	5/8	3/8
100 mμ	1/12	4/19	1/8	2/8

′ Not done.
″ Millipore membrane filters.

The experiments were repeated using only the filtrates which passed through the 0.45, 0.3 and 0.1 micron Millipore filters. Each of these filtrates produced lesions. The agent was recovered from each filtrate by inoculating embryonated

eggs. Further filtration trials were carried out in which filtrates from the 0.3 and 0.1 micron Millipore filters only were employed to inoculate seven-day embryonated eggs. Both filtrates produced death of the embryos, thus suggesting that the agent was less than 0.3 micron in size.

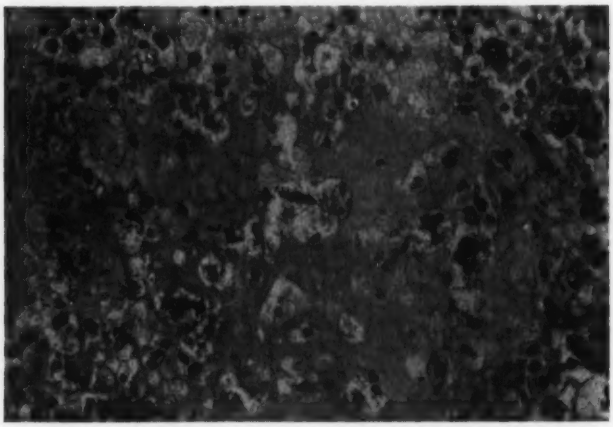

Fig. 4. Older lesion. All liver cells in the area have been destroyed and are replaced by proliferating reticuloendothelial cells and fibroblasts. A few lymphocytes may also be seen. Hematoxylin and eosin. 450×

DISCUSSION

The hepatitis of turkey poults reported here does not appear to be related to the avian infectious hepatitis recently reported in the literature.[1,2,4,5,6] Several isolations of a vibrio from chickens affected with a hepatitis were made in our laboratory following the technique described by Peckham[3], but when the same methods were applied to the hepatitis of turkeys, the results were negative.

In addition, the turkey hepatitis agent passed through a Seitz EK filter and through 0.45, 0.3 and 0.1 micron Millipore filters; it was also found to be resistant to penicillin, streptomycin and tetracycline (table 4).

In view of these findings it is our belief that the agent

TABLE IV

Resistance to antibiotics
Death pattern of chicken embryos inoculated at 6 days

Antibiotic used	Amount of drug per ml of inoc.	Embryo mortality— Days after inoculation Yolk sac route											No. deaths / No. inoculated
		1	2	3	4	5	6	7	8	9	10	11	
Tetracycline	5 milligrams						1	2	1	2	2		8/8
None	—					1		1	3	1		1	7/8
Penicillin	500 units				3	1	2						6/6
Streptomycin	1 milligram				6								6/6
None	—				1	3	1				1		6/6

responsible for the hepatitis described here is a virus, the incidence and economic importance of which remains to be established.

SUMMARY

An outbreak of disease exhibiting unusual mortality in turkey poults under two weeks of age was investigated in the spring of 1958.

A diagnosis of hepatitis was made. An infectious agent was isolated from the livers of affected birds by yolk sac inoculation in embryonated chicken eggs. The agent was maintained in eggs by the yolk sac route, but allantoic inoculation gave negative results.

The condition was reproduced in day-old poults by inoculating infective yolk or liver suspension into the unabsorbed yolk sac. Small gray necrotic foci appeared on the liver within seven days. Macroscopic examination revealed no other affected organs.

Aerobic and anaerobic cultures for bacteria and PPLO were negative. Filtration trials indicated that the agent was less than 300 millimicrons in size. It was resistant to penicillin, streptomycin and tetracycline. The effect of other chemotherapeutic agents is being studied.

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STUDIES ON PASTEURELLOSIS. III. CONTROL OF EXPERIMENTAL FOWL CHOLERA IN CHICKENS AND TURKEYS WITH AN EMULSIFIED KILLED VACCINE*

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IN a previous paper[1] it was shown that a high degree of immunity could be established against fowl cholera in chickens with highly concentrated killed *Pasteurella multocida* vaccines. A review of the literature was also presented at that time. The purpose of this study was to determine the lowest concentration of a suspension of formalin-killed *P. multocida* which would produce an immunity in chickens when incorporated in a water-in-oil emulsified vaccine.

MATERIALS AND METHODS

New Hampshire chickens and Beltsville Small White turkeys from closed flocks and free from all infectious diseases were used in these experiments. No chemotherapeutic agents, vaccines, or antibiotics were used in raising these birds.

Preparation of vaccine. A virulent culture of *P. multocida* (strain No. X-73, isolated from a peracute case of fowl cholera) was used to make the vaccine. The culture was suspended in skim milk and desiccated with a freeze-drying apparatus. When the culture was reconstituted, as described below, the colonies exhibited the following characteristics:

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circular (2 to 2.5 mm in diameter); smooth, convex and glistening; iridescent when observed through a stereomicroscope with obliquely transmitted light; butyrous, with tendency to coalesce. The colonies should be examined after 18 to 24 hours of incubation because older colonies exhibit different colonial characteristics. The organisms grew with a uniform turbidity in broth.

The seed culture for vaccine was prepared by reconstituting the desiccated stock culture in 1 percent peptone broth. This suspension was streaked on beef-infusion agar containing 5.0 percent chicken serum and was incubated for 24 hours at 37 C. The plates were held 24 hours before streaking in order to dry the condensate within the plates. Four or five colonies from a plate showing no dissociation (blue, sectored, or dwarf colonies) of individual colonies were used to inoculate 150 ml of peptone broth. The broth culture was incubated 6 hours at 37 C and shaken every hour to increase growth. Two and one-half to 3 ml of the seed culture were used to inoculate each Roux bottle, which contained 150 ml of dextrose starch agar. An additional 1.5 percent agar had been added to keep the medium from collapsing after inversion of the Roux bottles. After 24 hours incubation in an inverted position, the bacteria were washed from the agar of each Roux bottle with 10 to 15 ml of 0.25 percent formalized physiological saline and pooled in a large flask. A wire spreader used to spread bacteria for plate count is helpful in removing the bacteria from the agar.

Four bacterial concentrations, 100, 40, 20, and 10 x McFarland 1 density were prepared using 0.25 percent formalized physiological saline as the diluent. The suspensions were labeled lots A, B, C, and D, respectively. The number of *P. multocida* per ml when adjusted to a McFarland 1 density was approximately 1.3×10^9. A McFarland 1 density is equivalent to 79 percent light transmission on a Bausch and Lomb colorimeter (filter: 580 mu).

The bacterial suspensions were held overnight in a refrigerator or until all bacteria were dead. As a sterility test, 5 ml were removed from each lot and used to inoculate 2

serum agar plates, 2 tubes of thioglycolate broth and 50 ml of peptone broth enriched with 5.0 percent chicken serum. A similar test was made after the vaccine was bottled.

A volume of killed bacterial suspension was added to an equal volume of Bayol F* which contained 3 percent Arlacel A Special* and 0.1 percent formalin. The oil, emulsifier, and formalin were mixed in a Waring Blendor, and then the bacterial suspension was added while the blendor was running. For example, the following mixture was emulsified by processing in the blendor for 4 minutes:

243 ml of light mineral oil (Bayol F.)
7 ml of emulsifying agent (Arlacel A Special)
0.25 ml formalin
250 ml of killed bacterial suspension

The vaccine was bottled and labeled lots A, B, C, or D to correspond to the bacterial density from which it was made. The vaccine was stored in the refrigerator.

Vaccination and challenge. Each vaccinated bird was given 0.5 ml of vaccine injected subcutaneously in the midportion of the neck. Two hundred and thirteen 12-week-old chickens were divided into six groups. Groups 1, 2, 3, and 4 (37 birds each) were injected with 0.5 ml of vaccine A, B, C, and D, respectively. Group 5 (20 birds) was raised with the vaccinated birds and exposed as contact controls. Group 6 (45 birds) was raised in isolation and exposed as unvaccinated controls. Birds from each group, with the exception of group 5 which had no 26 weeks postvaccination challenge, were selected at 26, 36, and 54 weeks postvaccination and their immunity challenged by swabbing the nasal cleft with a suspension of *P. multocida*, strain P-936.

Thirty-one 28-week-old Beltsville Small White turkeys were divided into 2 groups. Group 1 (18 hens and 2 toms) was injected subcutaneously in the midportion of the neck with 0.5 ml of lot C vaccine. Group 2 unvaccinated controls

* The light mineral oil known as Bayol F was obtained from the Esso Standard Oil Company, Linden 1, New Jersey. The emulsifying agent, mannide monoleate, (Arlacel A Special) was obtained from the Atlas Powder Company, Wilmington, Delaware.

(11 hens) was raised in the same pen with the vaccinated group. The immunity of each turkey was challenged 21 weeks postvaccination.

A virulent culture of *P. multocida*, (strain P-936), which had been desiccated at the time of isolation, was used to challenge the immunity. When needed for challenge, the desiccated culture was reconstituted and streaked on agar plates as previously described for the vaccine strain. Smooth iridescent colonies were selected from plates showing no dissociation of colonies after 24 hours incubation at 37 C, and a suspension was made in peptone broth to a density of 79 percent light transmission. This was the base concentration for all dilutions of challenge. Approximately 2.9×10^7 organisms in 0.11 ml constituted the challenge dose. The dose was prepared by diluting the base suspension 1:5 with peptone broth and swabbing the nasopharynx of each bird with a cotton swab saturated with the bacterial suspension.

All birds were confined to 20 by 20 foot cinder block houses except the control group 6 which was raised in a 12 by 16 foot frame house with a grass runway. When birds were selected for challenge they were transferred to identical type cinder block houses with the exception of group 6 which was confined to finishing batteries in a separate isolation house. All birds that died were necropsied and cultured, and moribund birds were killed and also necropsied and cultured to confirm the diagnosis of fowl cholera as recorded in figure 1.

The palatine cleft of each bird was swabbed with cotton on a wood applicator at the conclusion of the experiments. The swabs were placed in tubes of peptone broth. After incubating for 3 to 4 hours, the broth cultures were streaked on dextrose starch agar plates, which were examined after 24 hours of incubation at 37 C for the presence of *P. multocida* colonies. The turkeys were observed for 10 weeks after exposure. Chicken exposed at 26, 36, and 54 weeks postvaccination were observed after exposure for 44, 34, and 19 weeks, respectively.

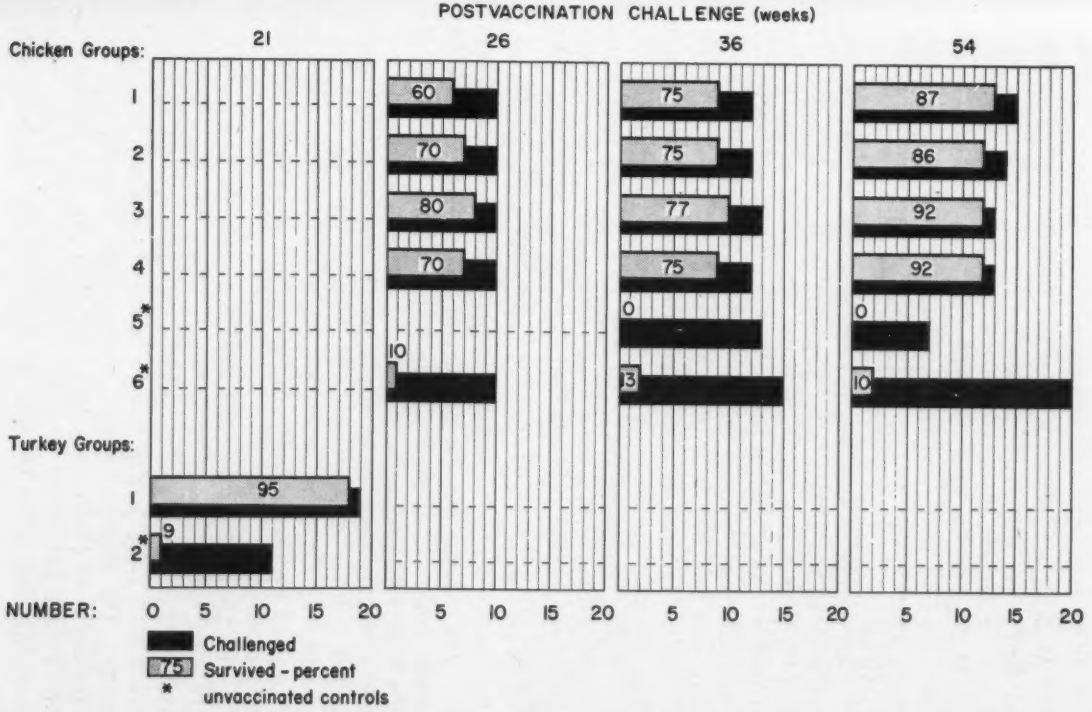

Fig. 1. Comparison of immunity produced by different concentrations of vaccine.

RESULTS

Analysis of the results in figure 1 indicates that there was no significant difference in the ability of vaccines of different concentrations to establish a high degree of immunity in chickens for at least one year. Eighteen of 19 (95 percent) turkeys that received lot C vaccine survived the challenge, whereas only 1 of 11 (9 percent) unvaccinated controls survived.

All deaths of unvaccinated chickens due to fowl cholera occurred within 2 days after exposure with the exception of two birds that died within 5 days. All deaths of vaccinated birds due to fowl cholera occurred within 14 days (average 7) after challenge with the exception of one bird that died at 81 days and one bird that died at 122 days after challenge. *P. multocida* was not recovered from the specimens taken from the palatine clefts at the conclusion of the experiment.

Four vaccinated chickens and one turkey died from various causes or lost wing bands before their immunity was challenged. Seven chickens died from causes other than fowl cholera after they were exposed to the challenge culture.

DISCUSSION

The reason for the proportion of survivors at the 54 weeks postvaccination challenge being higher than those at the 26 and 36 weeks challenge may have been due to environmental conditions. The 26 and 36 weeks exposures were made in hot weather during June and August. The 54 weeks exposure was made in December, when the temperature was down to 2 F. The immunity of the turkeys was also challenged in December.

By the methods now employed, it is difficult to determine the serological type of *P. multocida* which has been isolated from chickens. The same serological type is isolated from birds with fowl cholera and from the respiratory system of cattle. The strains isolated from cattle, however, will not produce fowl cholera nor will they stimulate the production of immune antibodies against the disease. Therefore, when selecting strains to be used in fowl cholera vaccines, only

those which were isolated from acute fowl cholera and which will produce the acute disease with at least 80 percent mortality should be considered. The colonies from such strains will usually exhibit the characteristics previously described for strain X-73. Any change in these characteristics is usually an indication of a reduced capsule, virulence, and immunogenic potency.

The lowest concentration of a suspension of *P. multocida* that would produce immunity, when incorporated in an emulsified vaccine, was not determined. It is the opinion of the writers, however, that a density of 10 x McFarland 1 is satisfactory. The vaccine should be injected subcutaneously in the middle third of the neck. In previous experiments (unpublished data) when the vaccine was injected on the head or at the base of the skull, the facial tissues swelled in some instances.

As was pointed out in the results, one of the vaccinated birds died 122 days after exposure. This indicates that the challenge culture survived in an immune bird or in the litter. The writers believe that the bird was a carrier of *P. multocida* for the 122 days. The danger from such a carrier lies in the possibility of transmitting the disease to susceptible birds that may be added to the flock.

SUMMARY

A study was made to determine the lowest concentration of a suspension of formalin-killed *Pasteurella multocida* that would produce immunity in chickens when incorporated in an emulsified fowl cholera vaccine.

Four groups of chickens were vaccinated with vaccine of different bacterial concentrations. Birds from each group plus unvaccinated controls were exposed to virulent *P. multocida* at 26, 36, and 54 weeks after vaccination. One group of turkeys was injected with one of the vaccines and their immunity was challenged 21 weeks after vaccination.

There was no significant difference in the ability of the vaccines of different concentration to establish a high degree of immunity in chickens for at least one year. Eighteen of 19

(95 percent) turkeys survived the challenge, whereas only 1 of 11 (9 percent) unvaccinated controls survived.

Criteria for selection of strains of *P. multocida* suitable for making vaccine and a method for production of the vaccine are presented.

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ASPERGILLOSIS EXPERIMENTALLY PRODUCED IN HATCHING CHICKS*

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A FAIRLY detailed search of the literature has revealed no record of experimental, incubator, brooder pneumonia infections. The results of a few limited experiments in this area are given below.

METHODS

Aspergillus fumigatus cultures were grown on wheat grains to obtain heavy sporulation. Wheat was washed three times to remove any fumigants and allowed to soak for 3 hours in tap water. The water was drained off and an amount of soaked wheat sufficient to cover the bottom of a 2000 ml flask

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to a depth of about one inch was used. The flasks were plugged with glass wool stoppers wrapped with cheese cloth, and autoclaved at 15 lbs pressure for 25 minutes. They were removed from the autoclave, shaken to loosen the wheat layer, and when cooled to room temperature, were ready for seeding. To 5 or 6 ml of sterile nutrient broth added 0.1 ml of a 1:10 dilution of sterile Lenconal wetting agent.** This mixture was generally applied to the *A. fumigatus* culture slant or Petrie dish culture by means of a 5 ml syringe and a 20 gauge 2 inch needle. The liquid containing the spores was aspirated and injected into the flask containing the sterile wheat. After shaking to insure a uniform distribution, the flask was incubated at 37 C for 5 to 6 days. Each day the flask was shaken to prevent the layer of wheat from becoming caked, to permit aeration and to promote sporulation on the entire wheat kernel.

The egg incubator used was a 400 egg capacity wooden case with bottom heating coil and a circulating fan at the top. Trays were covered on the bottom with 5/16 inch galvanized wire mesh.

The incubator was contaminated with 500 cc of *A. fumigatus* infected wheat placed on paper towels in the hatching trays. Four dozen single comb White Leghorn eggs, of 19 days incubation were placed in the machine at the same time. By the fourth day after hatching or six days after seeding the incubator, the chicks were necropsied.

RESULTS

Forty-two of the 44 chicks had air sac plaques, lung plaques or both. A second trial was made without cleaning or removing egg shells from the incubator. Thirty-six White Leghorn eggs at 19 days incubation were introduced for hatching. The birds were necropsied on the fifth day after hatching or the seventh day after the eggs were exposed. All showed fungus plaques in lungs, air sacs or both.

The incubator was now hosed with cold water and cleaned with a fiber brush. A group of 38 White Leghorn eggs that had been incubated for 19 days were placed in the cleaned

** Laboratory Equipment Corp., St. Joseph, Michigan.

incubator and held there for 12 days. No gross plaques were observed on lungs or air sacs and no microscopic indication of lung infection was found.

In another trial, the incubator was again seeded with the fungus and re-set with 108 (Vantress x White Rock) eggs and 108 ("Nichols" 88) eggs at 19 days of incubation. Mortality started by the first day after hatching in both groups. The mortality peak came for both groups between the 2nd and 3rd day after hatching. Plaques were found in all of the chicks which died. On the third day, the chicks were removed from the incubator and placed in identical brooders. By the 15th day of age, 87 of one group and 97 of the other had succumbed. All of these had plaques in the lungs, air sacs or both.

Into the same, uncleaned incubator were placed six 5-week-old chicks. These were exposed for four days, then removed to a cage. On examination 10 days later, no plaques were found.

This prompted a series of exposures in which eggs of 20 days incubation, 21 days and pipped, chicks 1 day after hatching, 2 days after hatching and 3 days after hatching were used. These were held in the seeded incubator for 5 days. A fungus infection in 80 percent or more of the chicks was obtained in all these groups up to and including that of the 2nd day after hatching. However, the 3-day-old group had an infection rate of only 40 percent. Apparently, within a few days after hatching, chicks rapidly develop increasing resistance to fungus spore infection. Further investigation seems indicated to ferret out this mechanism of resistance to fungus spore infection.

SUMMARY

Chicks in the process of hatching and up to two days of age were easily infected with *Aspergillus fumigatus* spores by contaminating the forced draft incubator with wheat which had been seeded with *A. fumigatus*. Chicks older than three days of age were resistant to infection.

TRANSAMINASES AND DEHYDROGENASES IN CHICKEN PLASMA*

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MEASUREMENTS of activity levels of enzymes in the blood have proven to be valuable diagnostic aids in human medicine. Elevated levels of glutamic oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), lactic dehydrogenase (LDH), and malic dehydrogenase (MDH) have been associated with certain pathological conditions. The possible significance of determinations of the levels of these enzymes as aids in the diagnosis of avian diseases has not been investigated. Cornelius, *et al.*, however, did observe elevated plasma GOT levels in chickens with inherited muscular dystrophy.[2]

ASSAY PROCEDURES APPLICABLE TO CHICKEN PLASMA

Many enzyme assay procedures have been described in the literature. Various methods were investigated in order to determine their applicability for chicken plasma. Qualities which were desired in the techniques included a rapid procedure with a minimum of manipulations, relatively stable reagents, and accurate, reproducible results.

The data presented in table 1 was collected by means of the methods found to be the most satisfactory. Commercially available transamination substrates were utilized. Transam-

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inase SGO,* which is adapted from the method of Karmen,[4] was employed for GOT determinations carried out on a Beckman DU spectrophotometer. The Sigma colorimetric procedure for measurement of GPT activity[6] was carried out on a Klett Summerson photoelectric colorimeter.

An adaptation of the method of Wroblewski and LaDue[9] using pH 7.5 phosphate buffer was employed for LDH assays. Reproducible values were obtained by the procedure both on a Beckman DU spectrophotometer (25 to 28 C. incubation temperature) and a Coenzometer** (36 C. incubation temperature). The latter instrument contains a fluorescent tube and has a single wave length of 350 mμ.[5] The sensitivity of the Beckman DU spectrophotometer is greater, and only the data obtained with this instrument is presented.

A modification of the method of Siegel and Bing[5] was used for MDH assays. The reaction solution contained 0.1 ml DPNH (2.5 mg/ml), 2.5 ml 0.1 M phosphate buffer pH 7.5, and 0.3 ml plasma. After a 20 minute incubation period (25 to 28 C.), 0.1 ml of 0.5 M oxalacetic acid was added. The optical density of the reaction was recorded at 60 second intervals for three minutes. Similar values for duplicate assays were usually obtained when this procedure was carried out at 340 mμ on the Beckman DU spectrophotometer. Occasionally a reaction would proceed in the wrong direction. This discrepancy could have been due to any of several factors. Therefore, more work is required on the analytical procedure for MDH activity in avian plasma.

ENZYME ACTIVITY LEVELS IN CHICKEN PLASMA

The activity levels of GOT, GPT, LDH, and MDH were measured in plasma samples from normal Single Comb White Leghorns (SCWL) and White Rocks (WR) reared in isolation units. The tests were conducted on the following age groups: 5, 6, 7, 8, 10, and 14 weeks, and 6 months. Heparinized blood samples were collected by cardiac punctures. A summary of

* Produced by Warner Chilcott, Morris Plains, N. J.
** Manufactured by Macalaster Bicknell Co., Cambridge, Mass.

the ranges of the levels of the various enzymes is presented in table 1. The ranges of the GOT and GPT activity levels were relatively constant in the various age groups so it was feasible to include all the values obtained in the calculations of the normal ranges. The average LDH activity level in 6-month-old birds, however, was much lower than the average levels in the younger birds. The average MDH levels in 14-week and 6-month-old chickens were also lower than the average levels of birds tested at earlier ages.

No significant statistical differences were detected between the average enzyme levels of sexually mature hens and roosters or SCWL and WR according to the unpaired "t" test.[7] Therefore, no differentiations were made in table 1 as to the breed or sex of the birds. Plasma samples were kept at –5 C. for 3 days without losing GOT activity and 7 days without losing LDH activity.

TABLE I

Plasma transaminase and dehydrogenase activity levels of normal chickens

Enzyme	Age of birds	No. birds tested	Enzyme activity units per ml plasma*		
Glutamic Oxalacetic Transaminase**	5 wk.-6 mo.	186	77	—	243
Glutamic Pyruvic Transaminase***	5 wk.-6 mo.	152	0	—	23
Lactic Dehydrogenase**	5 wk.-14 wk.	135	470	—	1,146
	6 mo.	30	205	—	689
Malic Dehydrogenase**	5 wk.-10 wk.	139	15	—	223
	14 wk.	30	0	—	128
	6 mo.	25	27	—	135

* Range = mean ± 2 S. D.
** One unit = amount of enzyme activity which will cause a decrease of .001 in optical density per minute under standard conditions.
*** Activity expressed as Sigma-Frankel units.

This data could not be compared with any found in the literature. Goswami and Robblee measured GOT levels in chick whole blood.[3] These levels would be expected to be higher than GOT levels in plasma because erythrocytes con-

tain considerable enzymatic activity. GOT levels in avian serum and plasma have been measured by colorimetric procedures.[1,2] However, these methods expressed the enzymatic activity in units which are different from those utilized in the assay procedures employed in the present investigation. Cornelius, *et al.* did not detect any GPT activity in serum from adult White Leghorns with a colorimeteric procedure.[1] No activity could be demonstrated in some of the plasma samples tested during this investigation also. Some GPT activity, however, was observed in most of the samples.

A preliminary survey was made of plasma transaminase levels in chickens afflicted with various diseases. The plasma samples were obtained from cases which were submitted to and diagnosed at the Department of Animal Pathology, University of Maine. A summary of the results is presented in table 2. No abnormal GPT levels were detected. Elevated GOT

TABLE II

Survey of plasma transaminase levels in abnormal chickens

Disease	No. birds tested	No. birds with high GOT levels	No. birds with high GPT levels
Avian encephalomyelitis	4	0	0
Coccidiosis	9	8	0
Infectious synovitis	3	2	0
Leukosis complex			
neural	2	2	0
ocular	1	1	0
visceral	8	5	0
Newcastle disease	35	0	0
Sulfonamide toxicity	4	1	0

GOT glutamic oxalacetic transaminase; GPT glutamic pyruvic transaminase.

levels were observed in some of the cases of coccidiosis, infectious synovitis, leukosis, and sulfonamide toxicity. These results are encouraging because the GOT levels were measured by the Sigma colorimetric procedure.[6] The sensitivity of this method for avian plasma is low due to a necessary 1:10 dilu-

tion of the plasma with distilled water. No conclusion can be made about the value of plasma enzyme determinations as diagnostic aids for avian diseases, however, before controlled experiments are conducted.

SUMMARY

Activity levels of glutamic oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), lactic dehydrogenase (LDH), and malic dehydrogenase (MDH) were measured in plasma from normal SCWL and WR from five weeks to six months of age. Assay procedures for these enzymes which are applicable to chicken plasma are discussed. The MDH procedure is not entirely satisfactory. Neither sex nor breed affected the enzyme levels. Age did not affect the ranges of GOT and GPT activities, but the average levels of LDH and MDH activities were lower in the older birds. In a preliminary survey, elevated GOT levels were observed in birds with various diseases.

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AVIAN ENTERIC CYTOPATHOGENIC VIRUSES† I. ISOLATION

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THROUGH the use of cell cultures, enteric viruses have been found in healthy and infected human beings[3,5,12] as well as animals.[1,4,6,7,8,10,11] To date, the isolation of such agents from avian sources has not been reported. It was felt that information on avian enteric viruses and their relationship to known as well as yet unrecognized diseases could be valuable, and with this in mind, attempts to isolate and study these agents were begun.

MATERIALS AND METHODS

A total of 103 specimens were collected from three groups of birds. Rectal swabbings were obtained from, 1) birds from a University of Connecticut Poultry Department flock exhibiting no clinical signs of disease, 2) specimens in poor health submitted to the Department of Animal Diseases Diagnostic Laboratory, and 3) birds of unknown health condition which were received at a poultry processing plant.

After the sample had been taken, the swabs were placed in sterile lusteroid tubes containing 8 ml of trypticase soy broth. After incubation at 4 C for 2 to 3 hours, the swabs were squeezed out and the entire fluid content of the tubes stored at –50 C until processed. When testing was begun, these tubes

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were thawed, the supernate withdrawn to a fresh, sterile tube, and this material used as the first passage inoculum.

Cell cultures. Calf, monkey and chicken kidney cell cultures were used in screening the samples. The calf cells were prepared in our laboratory according to the procedure outlined by Younger.[14] Monkey kidney cells* were made in a similar manner.

The chicken kidney cells were prepared by a modification of the method used by Bodian[2] for monkey cells. Kidneys from 1-week-old chicks were removed aseptically and placed in a 4 x 1 inch sterile test tube. They were then minced with sharp scissors until the larger pieces did not exceed 0.25 cm. After transfer of the tissue to a flask, washings in phosphate buffered saline[9] (PBS) were continued until the supernate was clear. The PBS was then replaced with 0.25 percent trypsin solution in the amount of 10 ml for each pair of kidneys. The flask containing the tissue pieces in trypsin was tightly stoppered and placed in a 4 C refrigerator overnight (16 to 18 hours).

The next morning, the flask and contents were shaken manually for approximately 3 minutes to dislodge the cells and cell clumps from the tissue matrix. All materials in the flask were then strained through four layers of sterile gauze, centrifuged for 10 minutes at 500 rpm using an angle head in an International Centrifuge, size 1, type SB. The packed cells were resuspended in fresh PBS and centrifuged again as described above. The growth medium, Hanks' basic salt solution[9] with 0.5 percent lactalbumin hydrolysate, and antibiotics (abbreviated as Hanks' + M), plus 10 percent calf serum was added to the sediment at the rate of 80 ml per pair of kidneys.

The cells were kept in suspension by a magnetic stirring bar and plate while 1 ml amounts were distributed into 16x 150 mm screw cap tubes by use of a Cornwall syringe. These tubes were then sealed with rubber lined plastic caps, placed

*The monkey kidney cells were supplied through the courtesy of The Section of Epidemiology and Preventive Medicine, The Yale University School of Medicine, New Haven, Connecticut.

in racks which held them approximately at a 5 degree slant, and incubated at 37 C. When bottle cultures were desired, 8 ml amounts were placed in sterile 8 oz. prescription bottles and then sealed with white rubber stoppers. In either case, confluent sheets were obtained 4 to 6 days after seeding.

Before inoculation, all tubes were changed to their respective maintenance media: the calf cells to Hanks' + M containing 2 percent horse serum, and the chicken kidney cells to Hanks' + M with 10 percent calf serum. All serum used in the maintenance media was inactivated by holding it at 56 C for 30 minutes.

Embryonated eggs. Eggs employed in these tests were obtained from the Department's supply flock. Ten-day-old embryonated eggs were inoculated via the chorioallantoic sac (CAS) and 7-day-old eggs via the yolk sac (YS).

Isolation procedure. The fecal samples, prepared as described, were inoculated into eggs as well as calf, monkey, and chicken kidney cells. The amount of the inoculum for each passage was maintained at 0.2 ml. The respective fluids, yolk (Y) and allantoic fluid (AF), were used as material for repassage in eggs, and both the supernate and cells adhering to the glass were harvested for repassage in the case of the cell cultures.

Pathogenicity tests in chicken embryos. The fourth cell culture passage of each enteric virus was inoculated into eggs via the CAS and YS routes. This was done to see if passage through cell cultures might enhance the pathogenicity of these agents for embryonated eggs.

RESULTS

Isolations. In the first passage, tests conducted in eggs resulted in only three samples producing deaths in 50 percent or more of the eggs from either route. The first passage in chicken kidney cell cultures, however, showed at least seven samples which produced suspicious effects in the cells, and three which caused definite and distinct cytopathogenic effects (CPE). No such changes were observed when the 103 samples were carried through two passages in calf and monkey cell

cultures. The second passage of the samples through chicken kidney cells revealed 19 samples producing CPE, and by the third passage, 45 samples were positive.

Only those samples which were positive in cell cultures were carried through a second egg passage. Of the 45, only one produced deaths in 50 percent or more of the embryos inoculated, and this sample had already shown CPE in cell cultures. As a result of these tests, egg passages were no longer continued in an attempt to detect enteric viruses from chicken fecal samples.

The effect of cell culture adapted viruses on embryonated eggs. When the fourth cell culture passage of positive samples was inoculated into eggs, 23 of the 45 (51 percent) produced deaths in 50 percent or more of the eggs inoculated. Of these, 6 (13 percent) produced deaths in both routes, 14 (31 percent) in YS only, and 3 (7 percent) in CAS only.

Examination revealed that 14 samples produced lesions in 50 percent or more of the embryos. One caused no deaths or lesions when inoculated by the YS route, and no deaths, but enlarged spleens when inoculated by the CAS route. A second sample caused deaths when inoculated by either route, and embryos from the CAS route were dwarfed. The remaining 12 samples, all from the Poultry Department flock, showed less variation in death pattern. Heart nodules were observed in each of these cases. Eleven of these 12 samples produced lesions in embyros inoculated by the CAS route, and only 1 did so in those inoculated by the YS route.

The effect of source of sample on percent of isolations. Some positive samples were obtained from each of the three groups of birds tested (table 1). Isolations were made from seven of 16 (45 percent) processing plant samples, 21 of 50 (42 percent) from the Poultry Department flock, and 17 of 37 (46 percent) from the diagnostic laboratory samples. It can be seen that the percent of isolations was not influenced by the source from which the samples were taken.

Effect of age of bird on percent of isolations. When the results were tabulated according to age of the birds, a marked

correlation became evident (table 2). From birds 17 weeks of age or younger, 41 of 74 samples were positive. This represents 55 percent isolation from this age group, 91 percent of the total isolations made, and 40 percent of the total samples tested. Those samples which were obtained from birds 5 through 8 months of age showed positive isolations in 2 of 12 cases. Expressed in percentages, this represents 16 percent isolations from this group, 4 percent of all the isolations made, and 2 percent of the total samples tested. Of samples tested from the group ranging in age from 9 through 12 months, the same number of isolations were made from an equal number of birds. There were only 5 specimens obtained from birds over 1 year of age, none of which were positive.

TABLE I

Effect of source of sample on percent of isolations

Source of sample	Number samples in group	Number positive isolations	% isolations in group	% total samples tested
Processing plant	16	7	44	7
Poultry Department flock	50	21	42	20
Diagnostic service	37	17	46	17
Totals	103	45		

TABLE II

Effect of age of birds on percent positive isolations

Age (months)	Number of samples		% isolation in relation to		
	Tested per group	Positive per group	Number samples per group	Number isol. made (45)	Number samples tested (103)
0 - 4	74	41	55.4	91.1	39.8
5 - 9	12	2	16.7	4.4	1.9
10 - 12	12	2	16.7	4.4	1.9
Over 12	5	0	0.	0.	0.
Totals	103	45			

DISCUSSION

Cultures of chicken kidney cells proved most useful in screening avian fecal samples for enteric viruses. Hanks' basic salt solution, enriched with lactalbumin hydrolysate and calf serum made a satisfactory growth and maintenance medium. Though inoculations of embryonated eggs did not seem as productive a method of isolating enteric viruses, death patterns and embryonic lesions did prove useful in grouping some of the cell culture adapted agents. For this reason, it was felt that the combination of these two methods was more useful than either one alone.

The fact that a similar percentage of isolations was made from birds in good health as well as those diseased, may indicate that such agents are common residents of the intestinal tract, and could cause disease under advantageous circumstances. The studies showed, however, a high correlation between the age of birds and the percent of isolations made. Such results are similar to those obtained with studies of enteric viruses in man[5,12,13] and animals,[7,8] where younger members of a population harbor the agents and, with advancing age, show an increase in immunity coupled with a drop in the rate of shedding these agents.

Though no isolations of enteric viruses were made using egg inoculations, cell culture passages were found to have increased the pathogenicity of some of the agents for embryonated eggs. Those which produced marked effects in eggs were obtained from the Poultry Department flock samples, and those producing the most severe lesions in addition to deaths in the embryos were from samples taken during the latter part of the sampling period. This emphasized the point that both cell cultures and embryonated eggs can be used to advantage in grouping for further identification those enteric agents isolated by cell cultures alone.

These experiments have established the existence of avian enteric viruses. The indentification of the agents isolated, though not completed at present, may provide information which will be useful in understanding some poultry diseases.

Having shown that these agents do exist may also be important as a link in disease studies of cyclic and cross infections among man, animals, birds, and insects.

SUMMARY

Using chicken kidney cells, avian enteric viruses have been isolated from healthy as well as diseased chickens. Isolation attempts using embryonated chicken eggs, calf or monkey cell cultures were unsatisfactory. Of the isolations made, 91 percent came from birds 17 weeks of age or younger. Pathogenicity tests in eggs using tissue culture adapted material indicates that more than one group of agents were isolated.

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AVIAN ENTERIC CYTOPATHOGENIC VIRUSES†
II. CHARACTERISTICS OF A PROTOTYPE

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IN a paper concerning preliminary experiments, the isolation of enteric virsuses from chickens was reported.[1] Of the isolations made from the 103 fecal samples tested, one agent was selected as a prototype and studied. The selection was based on its rapid and distinctive cytopathogenic effect (CPE) on chicken kidney cell cultures (figure 1), as well as its pathologic effect on embryonated eggs. This agent, enteric virus #89 (EV-89), was one of a group of isolates obtained from samplings of a University Poultry Department flock. This paper describes the series of experiments designed to group and, as far as possible, characterize these agents by using the prototype virus as a reference.

MATERIALS AND METHODS

Isolates. The isolates used in these tests were the fourth

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and fifth passage of chicken kidney cell culture adapted avian enteric cytopathogenic viruses isolated from a clinically negative University Poultry Department flock. The fecal swabs used to obtain these isolates were taken from the same 10 birds beginning when they were 4 weeks old and continuing for 5 consecutive weeks.

Cell cultures. Both calf and chicken kidney cell cultures were prepared in the laboratory as previously described.[1] The duck kidney cells, though prepared elsewhere,* were made in a similar manner.

Embryonated eggs. All eggs used in these tests came from the Department of Animal Diseases' supply flock.

Experimental animals. The wet chicks employed in the pathogenicity tests came from hatches of eggs from the supply flock. The 24-hour-old white mice were obtained from our breeding stock.

Viruses. In addition to EV-89, laboratory strains of cell culture adapted infectious bronchitis (IB), and avian encephalomyelitis (AE), as well as egg propagated Newcastle disease (ND) viruses were used in these tests.

Antisera. In the neutralization tests conducted, antisera for IB, AE, ND, and infectious laryngotracheitis (IL) were included. Hyperimmune serum for EV-89 was prepared by serial, intravenous inoculations of rabbits whose preinoculation blood samples did not reveal any naturally occurring antibodies for this agent.

Serum neutralization tests. All serum neutralization tests were conducted with cultures of chicken kidney cells. The first series of tests were those to determine the antibody status of the serum from the rabbits. Varying dilutions of the serum pool were made using Hanks' +M.[1] These dilutions, in 0.5 ml quantities, were incubated for 30 minutes at room temperature with 100 $TCID_{50}$ of EV-89. Both virus titration and the serum-virus mixtures were then inoculated into the cell cultures. In similar tests designed to attempt identification of

*Duck kidney cells were supplied through the courtsey of The Section of Epidemiology and Preventive Medicine, School of Medicine, Yale University, New Haven, Connecticut.

this agent, EV-89 versus IBV, ILT, NDV, and AEV immune sera were tested in the same manner except that the sera were not diluted.

Pathogenicity tests in wet chicks. The fifth cell culture passage of EV-89 was inoculated into newly hatched chicks. These chicks were divided into groups to provide for intracerebral, intraperitoneal, and intramuscular routes of injection and an uninoculated control. The intracerebrally inoculated chicks received 0.03 ml, and 0.1 ml was administered to each of the intraperitoneal and intramuscular inoculates.

Pathogenicity tests in suckling mice. The sensitivity of suckling mice to EV-89 was tested by inoculating intracerebrally 0.02 ml of undiluted fifth cell culture passage of this virus. The mice were observed during 2 passages, each of one month's duration. Material for repassage was obtained by skinning the mice and removing the brain and neck muscles. This material was ground with mortar and pestle and then suspended in sterile saline in a weight to volume dilution of 1:10.

Heat stability tests. In a report from the Texas Agricultural Experiment Station[2], differentiation of the chicken embryo lethal orphan (CELO) virus from several strains of infectious bronchitis virus was accomplished by heat tolerance tests. This method was applied to EV-89 and the cell culture adapted IBV. Both agents were incubated at 56 C. Periodically, small samples were withdrawn and inoculated into chick kidney cell cultures to test for CPE.

Hemagglutination (HA) tests. HA tests were made on allantoic fluid from eggs inoculated with the fifth cell culture passage of EV-89. Similar tests were conducted using the supernatant fluid from the fifth chicken cell culture passage. Saline as well as NDV infected allantoic fluid were included as controls. To each of these four test materials, 0.1 ml of a 1.5 percent saline suspension of washed chicken red blood cells was added to 0.1 ml of the test material. After mixing the test samples, the plate was rotated for 5 minutes, during which time observation was made for the occurrence of hemagglutination.

RESULTS

Serum neutralization tests. Since EV-89 was obtained from a clinically negative Poultry Department flock, all other isolates from this group were tested using EV-89 rabbit prepared antiserum. None of the 13 agents obtained in the first 3 weeks of sampling were neutralized. Of the three isolates obtained from samplings taken during the fourth week, two were neutralized, one of which was EV-89. All five isolates collected during the 5th week of sampling were neutralized when combined with this same hyperimmune serum (table 1.)

Antisera prepared against IBV, ILV, NDV, and AEV had neutralization indexes of 10^3 or greater for their homologous viruses. When used undiluted in these neutralization tests, none of the first three were able to prevent destruction of the cell sheet by EV-89. When the antiserum from chickens immunized with AEV was used, however, it was found to

TABLE I

Samplings from Poultry Department flock showing repeated isolations and agent groupings

Bird number	Age of birds in weeks				
	4	5	6	7	8
1	—	U	—	U	X
2	U	U	—	—	—
3	U	—	—	—	X
4	—	—	—	—	—
5	U	—	U	—	X
6	—	—	—	—	X
7	U	U	—	—	—
8	—	U	—	X	X
9	U	U	—	—	—
10	U	—	U	X	—

U = Agents other than EV-89
X = EV-89 type agents
— = No isolations

neutralize this agent. Preinoculation serum samples from the AE immunized birds did not show any such ability. In reverse tests, using the EV-89 rabbit antiserum against the tissue culture adapted AEV, the avian encephalomyelitis virus was completely neutralized.

Pathogenicity tests of EV-89. 1. *Cell cultures.* Though

no CPE was observed when the 103 samples were carried through two passages in calf and monkey cells, the fourth chicken kidney cell culture of EV-89 was again inoculated into these two cell types. This was done to see if adaptation to one kidney cell type might have increased the pathogenicity of the agent for other kidney cell cultures. Three rapid passages were completed with no observable alterations in either type of cell culture. Tests were made for the presence of the virus by three additional passages through chicken kidney cells.

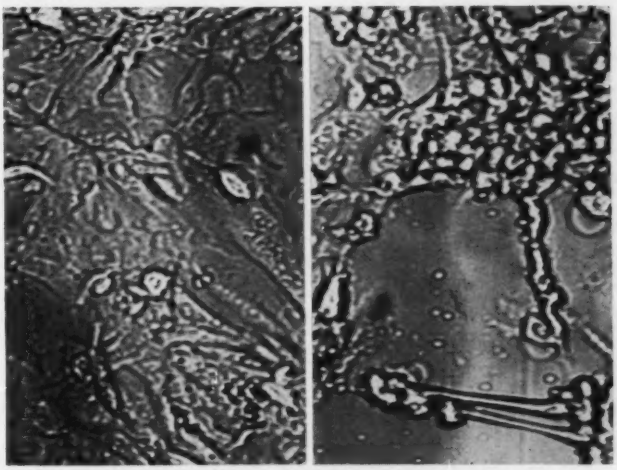

Fig. 1. Tube cultures of chicken kidney cells. (left) Uninoculated control. (right) Example of CPE produced 48 hours after inoculation of EV-89. 150×

No CPE was observed. When the duck kidney cultures were inoculated with the fourth cell passage of EV-89, CPE was produced in 48 hours. The effect of the virus on these cells was indistinguishable from that produced in chicken kidney cells.

2. *Chicken embryos.* The specific effects of EV-89 on embryonated eggs was studied by inoculation of undiluted fourth cell culture material via CAS and YS routes. The highest concentration of deaths occurred during the 15th through 17th day of development regardless of the route of inoculation. The greatest number of deaths occurred in the CAS inoculates

(66 percent) compared to 38 percent in the YS inoculated eggs. Fifty percent of those from the CAS group showed nodular hearts whereas only pale hearts, with slightly irregular outlines, were observed in the YS inoculated embyros.

3. *Wet chicks.* Two passages of EV-89 through newly hatched chicks caused twisting of the head and neck, and retropulsion in six of 20 inoculated via the intracerebral route. No such effects were produced during one trial where the chicks were inoculated by intramuscular and intraperitoneal routes.

4. *Suckling mice.* Pathogenicity tests in suckling mice were negative, although 2 passages, each of one month's duration, were conducted.

Plaque formation. Plaques were formed when EV-89 was inoculated into bottles containing sheets of chicken kidney cells (figure 2). By 21 days post inoculation, the plaques had attained a uniform size of 0.6 to 0.8 cm in diameter. The virus titer determined by the plaque method was 10^8 plaque forming units per ml whereas in tubes it was $10^{7.5}$ tissue infective doses per ml.

Heat stability tests. After incubation of EV-89 in a 56 C water bath, samples withdrawn at intervals to 22 hours still produced CPE in cultures of chicken kidney cells. The cell culture adapted infectious bronchitis virus included as a comparison did not produce CPE after being in the water bath for 30 minutes.

Hemagglutination tests. No agglutination of chicken RBC's was observed when tests were conducted with allantoic and chicken kidney cell culture fluids infected with EV-89.

DISCUSSION

With the EV-89 immune serum, neutralization tests of all the agents obtained from the Poultry Department flock indicated the presence of at least two agents or groups of agents. All but one of those isolated during the last 2 weeks of sampling were neutralized by EV-89 antiserum, while those isolated earlier were not. Possible explanations are that the second group of agents represented a new infection spreading through the flock, or that when immunity developed to the

Fig. 2. Bottle cultures of chicken embryo kidney cells. (left) Uninoculated control. (right) Inoculated with a 10^{-5} dilution of EV-89. Diameter of plaques, 0.3 to 0.4 cm by the 12th day post inoculation. 1×

first group, the second group was unmasked. The rise in the number of isolations from one group was concomitant with the decline in isolations from the other.

Serum neutralization tests did not reveal any protection of the cell sheet when IB, ILT, and NDV antisera were tested with EV-89, although each of these antisera showed high neutralization indexes for its homologous virus. If EV-89 is related to any of these three diseases, strain differences could prevent this relationship being detected.

Neutralization of the AE virus by the EV-89 immune serum, and the neutralization of EV-89 by the AE immune serum certainly indicated the need for further studies of the relationship between these two agents. It is entirely possible that the AE virus may be a common inhabitant of the intestinal tract, and that the EV-89 is related to, if not the actual virus of avian encephalomyelitis.

No increased pathogenicity for calf or monkey cells was observed when they were inoculated with the chicken kidney cell adapted EV-89, nor did tests reveal the presence of the virus in these cultures after three rapid passages. Though no CPE was seen in either of the first two cell types, the duck kidney cells showed susceptibility equal to that of the chicken kidney cells. Since there was a serologic crossing between EV-89 and the cell culture adapted AE virus, and since EV-89 grew well in duck kidney cells, it is suggested that AE might also be propagated in duck kidney cells. With wild birds so often found associated with poultry flocks, studies of the enteric viruses in these birds might prove interesting if run simultaneously with checks of the enteric viruses shed by the flock. It is conceivable that an infection cycle between the two exists.

The retropulsion and severe twisting of the head and neck produced by intracerebral inoculations of EV-89 into wet chicks does not establish this agent as the cause of a neuropathologic disease, though these results are interesting in conjunction with other experimental findings.

SUMMARY

In tests using one prototype avian enteric virus and its hyperimmune serum prepared in rabbits, the following characteristics were established:

1. Seven of 21 isolates obtained from an apparently healthy flock of birds were neutralized by the hyperimmune serum prepared against the prototype virus, indicating that more than one agent or group of agents were present in this flock.

2. Birds infected with one type of agent had previously yielded samples positive for an entirely different type of agent.

3. Duck kidney cells were susceptible to the prototype virus on first passage though no adaptation to calf or monkey cells was observed after three passages.

4. The prototype virus was pathogenic for wet chicks and embryonated chicken eggs, but not for suckling mice.

5. Heat tolerance tests showed the agent to be viable when tested after an incubation period of 22 hours at 50 C.

6. No hemagglutinins were found in infected materials from eggs or cell cultures.

7. Plaques were formed when the agent was inoculated into bottles containing cultures of chicken kidney cells.

8. Neutralization of this agent occurred when avian encephalomyelitis immune serum was used, and conversely, the avian encephalomyelitis virus was neutralized by the immune serum of the prototype virus.

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SWINE SERUM AND CO_2 IN THE ISOLATION OF AVIAN PPLO*

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PREVIOUS work[3,4] at this and other laboratories had indicated that research with avian pleuropneumonia-like organisms (PPLO) was being hindered by the inefficiency of the presently available culture media. This series of studies was made in an attempt to find culture media that were better able to support the growth of "pathogenic"[2] PPLO and to aid in their separation from "non-pathogenic" PPLO. The approach used in this work was based on the observation by Dr. James G. Lecce[6] that certain porcine and avian strains of PPLO were very fastidious in their growth requirements and grew only on plates containing swine serum, streaked with *Staphylococcus*, and incubated in CO_2 (candle jar technique).

MATERIALS AND METHODS

Plating media. All plating media were prepared from Difco PPLO agar. The following abbreviations are used in this paper:

P = PPLO agar + 10 percent horse serum
HP = PPLO agar + 10 percent Horse Serum + 1 percent yeast hydrolysate†
SP = PPLO agar + 10 percent swine serum
SHP = PPLO agar + 10 percent swine serum + 1 percent yeast hydrolysate

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The horse and swine sera were prepared by centrifugation and Seitz EK filtration and kept frozen at –25 C until used. All the above media were supplemented by the addition of 1000 units of penicillin per ml and 1:2000 thallium acetate as inhibitors. A similar group of media made up without inhibitors was labeled P_1, HP_1, SP_1, SHP_1 respectively. All plates incubated in air were placed in a water-jacketed incubator at 37 C. All plates incubated in excess CO_2 were placed in candle jars. In one experiment, a series of plates were incubated in similar jars but without the use of candles.

Overlay media. Several modifications of Adler's[1] overlay media were used.

A = Adler's overlay media with 10 percent horse serum.

SA = Adler's overlay media with 10 percent swine serum.

Both of these media were used with added inhibitors as noted above and incubated under ordinary atmospheric conditions.

Carbohydrate media. Two modifications of Grumbles'[5] phenol red base carbohydrate media were used.

M = Phenol red broth base + 1 percent maltose + 10 percent horse serum + inhibitors

SM = Phenol red broth base + 1 percent maltose + 10 percent swine serum + inhibitors

These media were also incubated under ordinary atmospheric conditions and kept for 14 days before being discarded.

PPLO isolates. The PPLO cultures used in these experiments were media-adapted isolates of varying types (S_6 "pathogenic" and C "non-pathogenic"). Most of these strains have since been tested by serological techniques and found to be stable in their characteristics.

Field tissues. This material consisted of tracheas and air sacs collected from field cases of respiratory disease at the New York State poultry diagnostic laboratories at East Aurora, Ithaca, Kingston, and Oneonta.

Examination of plates. All plates were examined at intervals up to the 6th day of incubation with a binocular mi-

croscope at 50 diameters magnification. Colony diameters were measured with an ocular micrometer. In all experiments except the first, one ocular micrometer unit equalled 19.7 microns. In the first experiment, one ocular micrometer unit equalled 200 microns.

In some experiments, measurements were made of typical or average colony size. In some experiments, only the maximum colony size was recorded. In others both measurements were taken. It was found as the work progressed that the former measurement was more significant and reliable.

EXPERIMENTAL AND RESULTS

Experiment 1. The effect of swine serum, CO_2, and Staphylococcus streak on the growth of some PPLO cultures. Two sets of plates were used in this experiment: P_1 and SP_1 plates. One plate of each type was inoculated with each of 23 S_6 type isolates and 12 C type isolates. Each plate was then inoculated perpendicularly to the PPLO streaks with a *Staphylococcus* streak. The P_1 plates were incubated in air; the SP_1 plates in excess CO_2.

After 6 days of incubation, the plates were examined and the diameter of typical colonies was measured. The average diameter of the S_6 colonies was 0.14 micrometer units (range 0.1 to 0.3) on the P_1 plates and 0.96 micrometer units (range 0.6 to 1.4) on the SP_1 plates. The average diameter of the C colonies was 1.1 micrometer units (range 0.7 to 1.8) on the P_1 plates and 1.4 micrometer units (range 1.1 to 2.0) on the SP_1 plates. No evidence of satellitism of the PPLO colonies in relation to the *Staphylococcus streak* was noted on any of the plates.

This marked stimulation in growth of the S_6 type colonies on plates containing swine serum and incubated in CO_2 stimulated further work on the effect of these two factors. Only slight stimulation of the C type colonies occurred under the same conditions. Since the *Staphylococcus* streak had no noticeable effect on either type of colony, its use was abandoned.

Experiment 2. The effect of swine serum, CO_2, and yeast

hydrolysate on the growth of some PPLO cultures. Four sets of plates were used in this experiment: P, HP, SP, and SHP plates. Two plates of each media were inoculated with each of four S_6 type isolates and three C type isolates. One plate of each pair was incubated in air and the other plate was incubated in a candle jar. After 6 days of incubation, the plates were examined and the diameter of the largest PPLO colonies measured at 50× magnification with an ocular micrometer. The results are presented in table 1.

Marked stimulation of S_6 type colonies was produced by incubation in Co_2 as in the previous experiment. On the other hand, only a slight stimulation of S_6 type colonies was produced by the use of swine serum. Yeast hydrolysate produced

TABLE I

A comparison of PPLO growth on different plating media incubated with and without excess CO_2 (Experiment 2).

Medium	In micrometer units Average maximum diameter of S_6 colonies (4 strains)	Average maximum diameter of C colonies (3 strains)
P	4.0	13.3
P + CO_2	9.0	16.6
HP	3.5	39.3
HP + CO_2	6.0	40.3
SP	3.8	12.3
SP + CO_2	8.5	15.0
SHP	5.8	29.6
SHP + CO_2	8.0	37.0
Treatment		
All plates containing yeast hydrolysate	5.8	36.6
All plates without yeast hydrolysate	6.4	14.3
All plates incubated in CO_2	7.9	27.2
All plates not incubated in CO_2	4.3	24.6
All plates containing horse serum	5.6	24.9
All plates containing swine serum	6.5	24.5

P = PPLO agar + 10 percent horse serum
HP = PPLO agar + 10 percent horse serum + 1 percent yeast hydrolysate
SP = PPLO agar + 10 percent swine serum
SHP = PPLO agar + 10 percent swine serum + 1 percent yeast hydrolysate

a marked stimulation of C type colonies but had little effect on S_6 type colonies.

Experiment 3. The effect of swine serum, CO_2, yeast hydrolysate, and inhibitors on the growth of some PPLO cultures. This experiment was similar in design to the preceding experiment with the exception that a fourth variable, the presence or absence of inhibitors in the media, was introduced. Four S_6 type isolates and 4 C type isolates were used.

When the plates were examined after 6 days incubation, not only the maximum colony size was recorded for each strain but also the typical colony diameters were recorded. The typical diameter was arrived at by measuring individual colonies, well separated, which on inspection appeared to represent the average size colony on the plate. The results are presented in table 2. The effect of swine serum, CO_2, and

TABLE II

A comparison of PPLO growth on different plating media incubated with and without excess CO_2 (Experiment 3).

Treatment	In micrometer units			
	Ave. maximum diam. S_6 colonies	Ave. typical diam. S_6 colonies	Ave. maximum diam. C. colonies	Ave. typical diam. C. colonies
All plates containing yeast hydrolysate	11.8	2.4	24.9	11.7
All plates without yeast hydrolysate	8.0	1.8	11.4	6.6
All plates incubated in CO_2	13.0	2.7	21.0	9.4
All plates not incubated in CO_2	6.8	1.5	15.3	7.9
All plates containing horse serum	8.6	2.2	17.9	8.0
All plates containing swine serum	11.2	2.0	18.4	9.3
All plates containing inhibitors	10.2	2.2	17.3	7.5
All plates not containing inhibitors	9.6	2.0	19.0	9.8
Plates not containing yeast incubated in CO_2	12.6	2.9	25.3	9.6
Plates not containing yeast incubated in CO_2	9.8	2.5	12.5	6.1
Plates containing yeast not incubated in CO_2	6.8	1.7	19.4	8.2
Plates not containing yeast and not incubated in CO_2	5.0	1.2	8.2	4.1

yeast hydrolysate in this experiment confirmed the results presented in experiment 2. The added variable, the presence or absence of inhibitors, had no significant effect on the growth of either type of PPLO.

Experiment 4. The effect of incubation in air, moisture-saturated air, and CO_2 on the growth of some PPLO cultures. Since in the previous experiments all plates that were incubated in CO_2 candle jars also were exposed to an atmosphere saturated in moisture, these experiments were designed to test the effect of moisture-saturated air in the absence of excess CO_2.

a) A series of 24 S_6 type PPLO isolates were diluted to 10^{-3} in nutrient broth. One drop (0.03 ml) of diluted culture was inoculated onto the surface of each of three SP plates and spread with a sterile bent glass rod to insure even distribution of the colonies. One plate in each series was incubated in air, one plate in a candle jar, and one plate in a similar jar without CO_2. Due to incubator failure it was not possible to read plates at the usual 6 day period and only the 4 day readings were available. At this time PPLO were found on only 17 of the 24 plates incubated in air, while colonies appeared on 22 of the 24 plates incubated in moisture or in CO_2.

b) A similar experiment was repeated using 23 S_6 and 7 C type isolates. Each isolate was inoculated onto three plates and incubated in the same three manners. The plates were examined after 6 days of incubation. The results are in table 3.

TABLE III

The effect of incubation in various atmospheric conditions on growth of PPLO (Experiment 4b)

Incubation in	In micrometer units			
	Average maximum diam. S_6 colonies	Average typical diam. S_6 colonies	Average maximum diam. C colonies	Average typical diam. C colonies
Air	3.3	1.7	10.7	8.1
Moisture-saturated air	5.8	3.0	13.8	11.2
CO_2 and moisture-saturated air	7.9	4.0	18.6	14.3

These results indicated that although the presence of

moisture-saturated air stimulated the growth of PPLO colonies, the presence of excess CO_2 had an additional stimulatory effect. These effects were more marked with the S_6 type isolates than with the C type PPLO.

c) This experiment was conducted as a supplement to experiment b and used the same PPLO isolates. Each isolate was diluted 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} in nutrient broth. A loopful of each of these dilutions was inoculated onto a section of each of three plates and incubated under the same three conditions. The plates were examined after 6 days of incubation for the presence or absence of colonies. The results of this trial are shown in table 4.

The effects of moisture and CO_2 were similar to those noted in the previous experiment. In this case, however, the design of the experiment permitted a more accurate quantitative evaluation of the results and suggested the potential usefulness of the new techniques in isolation procedures.

TABLE IV

The effect of incubation in various atmospheric conditions on the reisolation of PPLO from diluted cultures (Experiment 4c)

Dilution of inoculation	S_6 type cultures incubated in			C type cultures incubated in		
	Air	Moisture saturated air	CO_2	Air	Moisture saturated air	CO_2
10^{-2}	0	0	0	0	1	0
10^{-3}	3*	0	0	2	1	0
10^{-4}	7	4	3	2	3	5
10^{-5}	9	10	5	3	2	2
10^{-6}	4	9	15	0	0	0

* The numbers in the body of the table refer to the number of isolates whose end-point of growth (measured by presence of colonies) was reached at this dilution.

Experiment 5. A comparison of the effectiveness of some media isolation techniques for the reisolation of PPLO from diluted cultures. Two similar experiments were conducted to evaluate the comparative effectiveness of several PPLO media. Two duplicate series of isolation procedures were used as indicated in table 5.

Each series of isolation procedures was done with 10^{-1} to 10^{-9} dilutions of three PPLO suspensions: S_6 type, C type, and a mixture containing equal amounts of S_6 and C.

After the PPLO dilutions were made, five tubes or plates of each isolation series were inoculated from each dilution of each suspension to secure sufficient data capable of statistical analysis and to allow reading in terms of an ID_{50}. In the first experiment (5a) the pathogen used was isolate 293 and the non-pathogen was isolate 8. In the second experiment (5b) the pathogen was isolate TXMAR (Texas C6) and the non-pathogen was isolate 3W. Otherwise the two experiments were identical.

TABLE V

Outline of isolation procedures used in experiment 5. Each passage was made after 3 days of incubation

Direct 1st passage	2nd passage	3rd passage	4th passage
A	SA	A	HP (in air)
HP (in air)	HP (in air)		M
M	M		
SA	A	SA	SP (in CO_2)
SP (in CO_2)	SP (in CO_2)		SM
SM	SM		

A = Adler's overlay medium + 10 percent horse serum
SA = Adler's overlay medium + 10 percent swine serum
M = Modified Grumbles' maltose medium + 10 percent horse serum
SM = Modified Grumbles' maltose medium + 10 percent swine serum
HP = PPLO agar + 10 percent horse serum + 1 percent yeast hydrolysate
SP = PPLO agar + 10 percent swine serum

The results of both experiments are summarized in table 6.

The attempts made in this experiment did not permit clear cut differentiation of the two PPLO types. Only on a very few plates was separation actually achieved. In the other media, the mixing of the two PPLO types gave variable results.

Experiment 6. A comparison of the effectiveness of various procedures for the isolation of PPLO from field cases of respiratory disease. Since the previous experiments were done with PPLO isolates that were already well adapted to media, this experiment was devised to compare the isolation

TABLE VI

A comparison of the efficiency of reisolation of different types of PPLO from diluted cultures by different techniques. The results are expressed as ID_{50}

Type PPLO	Medium	Experiment 5a Direct to media	Experiment 5a To media from 1st passage overlay	Experiment 5a To media from 3rd passage overlay	Experiment 5b Direct To media	Experiment 5b To media from 1st passage overlay	Experiment 5b To media from 3rd passage overlay
S_6	M	7.63	8.25	8.25	6.50	5.63	6.00
	SM	8.22	7.83	7.83	5.68	6.38	6.50
C	M	7.50	7.50	7.50	5.50	6.38	6.38
	SM	<1.0	<4.0	<4.0	<5.0	5.83	5.83
S_6+	M	7.63	7.68	7.68	6.38	7.17	7.17
C	SM	7.38	7.38	7.38	7.17	7.17	7.50
S_6	HP	3.50	8.38	8.38	<3.0	5.50	6.50
	SP	5.68	7.83	7.83	<3.0	6.63	6.83
C	HP	5.17	7.50	7.50	3.50	6.38	6.38
	SP	5.50	6.63	6.63	3.50	5.83	5.83
S_6+	HP (S_6)	2.50	7.68	7.50	<3.0	7.38*	7.38
C†	HP (C)	4.32	6.75	7.50	3.5	7.38*	7.17
	SP (S_6)	3.17	7.38	7.38	<3.0	7.50*	7.50
	SP (C)	4.63	7.17	7.38	3.63	7.50*	6.83

* In this series it was impossible to recognize separate colony types.

† In this section of the table the results of plating from the mixed PPLO culture are presented as a double set of figures representing the ID_{50} of each component of the mixture. In each case the titration was made on the basis of the absence or presence of typical colony types determined on the basis of size and growth rate of each of the plates.

M = Modified Grumbles' maltose medium + 10 percent horse serum
SM = Modified Grumbles' maltose medium + 10 percent swine serum
HP = PPLO agar + 10 percent horse serum + 1 percent yeast hydrolysate
SP = PPLO agar + 10 percent swine serum

procedures with field material. In this case not only the direct growth of the organisms could be compared but also the ability of the organisms to adapt to the various media. Isolations were attempted from 130 tracheas and air sacs submitted from the various New York State poultry diagnostic laboratories. Tracheal suspensions were made by swabbing out the trachea with a sterile cotton swab and suspending the material in 2 ml of nutrient broth. The air sac material was ground with 5 ml of broth in a Ten Broek grinder. Part of the untreated suspensions was inoculated into media as described in table 5 with the difference that no plates were inoculated on the first passage. The remainder of the suspensions was treated with 10,000 units of penicillin and 50 micrograms of streptomycin per ml of suspension. After incubation for one hour at room temperature, 0.2 ml of suspension was inoculated into the yolk sac of three 5 or 6 day chick embryos. The embryos were candled daily. Any dead embryos were harvested and inoculated into media as already described for the untreated portion of the inoculum. If no embryo died by the seventh day after inoculation, one was selected at random for harvesting and culture in media. Thus each tissue suspension was subjected to four procedures; two directly into media indicated H and S and two into media after one embryo passage indicated IH and IS.

Isolations were attempted from 130 tissues coming from 65 accessions. Isolations were made from 40 tissues coming from 32 accessions. In 31 cases where tracheal and air sac suspensions were used from the same bird, PPLO were isolated from 13 tracheas and only three air sacs.

Table 7 compares the results of the four procedures. A preliminary classification of the PPLO type isolated was made on the basis of colonial characteristics (size and growth rate). This classification was later confirmed serologically with a great majority of the strains.

The use of swine serum and CO_2 significantly improved the isolation of S_6 strains of PPLO. However, as noted in previous reports, the use of embryos as an enrichment medium gave still better results than any procedure using artificial

media alone. It should be noted that comparison was made in this experiment only of horse serum plates incubated in air and not in CO_2.

TABLE VII

A comparison of the effectiveness of four procedures for the isolation of PPLO from field cases of respiratory disease.

	Procedure			
	H	S	IH	IS
S_6 cultures isolated	2	9	8	21
S_6 & C cultures isolated	8	9	7	9
C cultures isolated	5	4	3	3

H = Direct passage through media containing horse serum, plates incubated in air.
S = Direct passage through media containing swine serum, plates incubated in excess CO_2.
IH = Same as procedure H after one embryo passage.
IS = Same as procedure S after one embryo passage.

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SUMMARY

1. A series of experiments was carried out comparing the effect of some media ingredients and atmospheric conditions on the growth of PPLO colonies on agar plates. Both "pathogenic" S_6 and "non-pathogenic" C types of PPLO were used in these tests. The following conclusions were reached.

a) Moisture saturated air is advantageous for PPLO growth.

b) The presence of excess CO_2 in the atmosphere stimulates PPLO growth. This stimulation is more pronounced with S_6 than with C types of PPLO.

c) Swine serum stimulated S_6 type PPLO slightly more than horse serum.

d) Yeast hydrolysate had little effect on S_6 PPLO but markedly stimulated C type PPLO.

e) The presence or absence of inhibitors (penicillin and thallium acetate) had no effect on the growth of either type of PPLO.

f) No stimulatory effect or evidence of satellitism was noted when a *Staphylococcus* streak was used on plates inoculated with PPLO.

2. Two experiments were carried out to test the effectiveness of some media isolation techniques for the reisolation of PPLO from diluted cultures. In each experiment a pure S_6, a pure C type, and a mixture of the two types were used as starting material. No consistent differentiation in behavior of the two types of organisms was obtained. There was little indication under the conditions of this experiment that these techniques would offer a useful method of separating the two types of organisms from a mixed culture.

3. An experiment was conducted to compare the effectiveness of various procedures for the isolation of PPLO from field cases of respiratory disease. In this experiment the use of swine serum in the media and an excess of CO_2 in the atmosphere significantly improved the isolation of S_6 strains of PPLO. However, the use of embryos as an enrichment medium gave better results than any procedure using media alone.

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SEROTYPES OF *SALMONELLA* AND ARIZONA ORGANISMS IN ANIMALS: 1958†

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THE following outline is designed to help diagnosticians in processing materials for the isolation and identification of *Salmonella* and Arizona organisms.

SELECTING CULTURES FOR *SALMONELLA* SEROTYPING

Isolation. 1. Internal organs. Infusion agar (beef or horse) at a pH of 7.2 to 7.4 is ideal for direct culture from infected tissue, except of course, in the case of *S. pullorum* which grows slowly and scantily on this medium, but which can be cultured readily on tryptose agar. When it is advisable to use an enrichment broth, and when it seems reasonable to assume that the specimen to be cultured is not grossly contaminated with other organisms, infusion broth makes a satisfactory enrichment. Specimens suspected of contamination should be handled like intestinal contents as described below.

2. Intestinal contents. For the worker in the field of animal diseases who has no reason to be concerned with the isolation of *S. typhi* nor members of the genus *Shigella*, Kauffmann's tetrathionate enrichment medium, containing bile and brilliant green, and Kauffmann's brilliant green agar make a satisfactory combination for isolation of *Salmon-*

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ella from intestinal contents, particularly if these media are made up in the laboratory, rather than purchased as commercially-made dehydrated media. Plates should be held for 3 days before discarding.

Screening. Suspected colonies should be picked to TSI (triple sugar-iron agar) slants. In the case of Kauffmann's Brilliant Green Agar, *Salmonella* colonies, being non-lactose fermenters, are pink in color; so too, are many of the organisms in the Arizona group. In cases where there are no pink colonies, it would be well to pick one or more of the yellow or green colonies for the reason that some of the Arizona organisms ferment lactose rapidly, and if all lactose-fermenters are automatically discarded, members of this group of pathogens may be overlooked. The butt of the TSI slant is stabbed first, then the surface of the slant is streaked. *Salmonella* typically produce such abundant H_2S that the entire butt of the medium is blackened. The surface of the slant will be alkaline. Typical growth should be inoculated into the following media and held for four days: peptone water, glucose broth, Simmon's citrate agar, Christensen's urea agar, and lactose, sucrose, glucose and salicin broths. Cultures giving the following reactions should be investigated further:

Indole	M-R	V-P	Citrate*	Urease	H_2S*	Glu.	Lac.	Suc.	Sal.
—	+	—	+	—	+	AG	—or A	—	—

Generally speaking, organisms with the above-mentioned characteristics will fall into one of these groups: *Salmonella*, Arizona or Citrobacter. The Citrobacter group is KCN-positive and can be eliminated with Moeller's test. Arizona and *Salmonella* cultures can usually be differentiated by these additional tests:

	Jordan's tartrate	Dulcitol	Malonate	Gelatin liquefaction
Salmonella	+	AG	—	—
Arizona	—	—	+	+

Submitting cultures. A reasonable amount of information should accompany the cultures: the species of animal from which isolated, source of the cultures (heart, cecum etc.), and if there are several cultures from the same individual or flock,

* A few *Salmonella* types are H_2S or citrate negative.

this should be noted. Cultures should be on firm agar in screw-cap tubes. Packaging should conform to postal regulations, and addressed to: Enteric Laboratory, Box 185, Chamblee, Georgia.

SALMONELLA AND ARIZONA CULTURES FROM ANIMALS: 1958

During 1958, 1297 cultures from animals were serotyped in the enteric laboratory at the Communicable Disease Center, Chamblee, Georgia. There were 1178 members of the genus *Salmonella* and 119 belonged in the closely related Arizona group:

	Salmonella	Arizona	Combined
Turkeys	57.1	74.7	58.7
Chickens	28.6	4.2	26.3
Other birds	1.5	0	1.4
Total avian	87.2	79	86.5
Other farm animals	7.5	4.2	7.2
Household pets	1.4	0	1.3
Rodents	1.6	0	1.5
Other mammals	2	10	2.7
Reptiles	0	6.7	0.6
Total (other than avian)	12.7	21	13.4

Of 53 different *Salmonella* serotypes encountered, 40 were found in turkeys, 32 in chickens, 18 in all other avian species, and 24 in all other animal species. *S. typhi-murium* was the most widely distributed serotype.

There were nine different Arizona types encountered from six different animal species. The large percentage of cultures from turkeys includes but two serotypes.

In addition to the 1297 cultures from animals, 558 cultures were received from processed products of animal origin designed for either human or animal consumption.

CONTROL OF CHRONIC RESPIRATORY DISEASE I. THE EFFECTS OF CHLORTETRACYCLINE (CTC) AND TEREPHTHALIC ACID (TPA)†

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ALTHOUGH chronic respiratory disease (CRD) flocks have been established experimentally, birds free of CRD are not available to most hatcheries. Researchers commonly agree that *Mycoplasma* (MP) contribute to the development of the CRD complex. If some means could be discovered to control the various strains of MP, major progress in the control of CRD would be accomplished. Therefore, some means of controlling or eradicating MP and the losses resulting from CRD is urgently needed.

Fahey and Crawley[1] reported that chlortetracycline (CTC) 200 gm per ton of feed reduced the mortality, improved feed conversion and retarded or completely suppressed the MP antibody response. Olesiuk, *et al.*[3] fed 600 gm CTC per ton of feed for 14 days post injection and found a slight decrease in signs and lesions of CRD. Hofstad[2] believed that treatment with CTC (200 to 400 gm per ton of feed) was

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costly and of doubtful value.

Peterson[4] showed that terephthalic acid (TPA) potentiated CTC approximately four times. This was confirmed by Shelton and Olson[6] using infectious synovitis in chickens as a biological test organism. With the use of TPA, CTC may prove to be of value in the treatment of CRD and also may contribute to the development of an eradication program.

In broiler flocks, medication is useful even though the infection is not eliminated as long as the birds make a satisfactory weight gain and feed conversion and there is a satisfactory livability. If a medication is to be used in an effective eradication program then it is necessary for the causative agent to be eliminated from the bird.

This report includes three experiments conducted under field conditions to study the effects on CRD of CTC when provided continuously in the ration at 125, 250, or 375 grams per ton with and without 0.5 percent TPA, and the effect of feeding 250 gm per ton CTC plus 0.5 percent TPA for 2, 4, or 9 weeks.

It has been shown that the level of calcium and magnesium in the ration affects the efficacy of the tetracycline antibiotics.[5,7] The calcium and magnesium content of the ration in trial 3 was checked by chemical determination and was 1.13 percent calcium and 0.27 percent magnesium. The rations used for trials 1 and 2 were prepared according to the same formulation as for trial 3 except different batches of ingredients were used.

MATERIALS AND METHODS

Available for these experiments was a 20 by 80 foot house divided by solid wooden partitions into 20 by 20 foot pens, each equipped with removable wire partitions to permit subdivision into 10 by 20 foot pens. In each of these eight pens, 200, 180, or 100 chicks were started in trials 1, 2, and 3 respectively. An equal number of control birds were reared in batteries in another building under isolated conditions. Approximately 4,300 birds were used.

In trials 1 and 2, except the controls, the chicks were

vaccinated for Newcastle disease and bronchitis at 2 weeks of age and 25 percent of the birds were inoculated into the sinus with 0.25 ml of a 72 hour broth culture or an embryo culture of *Mycoplasma* (WVU 1791). Chlortetracycline was started at day old and fed continuously at 0, 125, 250 or 375 grams per ton of feed without and with 0.5 percent TPA.

In trial 3 the chicks were vaccinated for Newcastle disease at 2 weeks and for bronchitis at 4 weeks. All chicks, except the controls, were inoculated with a 3 day broth culture of MP (WVU 1791) at day old with one drop intranasally. Chlortetracycline was fed at 250 gms per ton of feed plus 0.5 percent TPA. The medications were given to duplicate pens and begin as follows: on the 4th day and continued through 9 additional weeks and on the 14th day and continued for 2 or 4 weeks.

All birds were blood tested for CRD at 8 or 9 weeks using the whole blood plate test in trial 1 and the serum plate test in trials 2 and 3. During trial 3, more frequent tests were made on approximately 20 percent of the birds in each pen. These tests were conducted when the birds were 5 days, 2, 4, and 6 weeks of age. In trial 1, 10; trial 2, 20; and trial 3, 20 or 100 trachea swabs were cultured for *Mycoplasma* at the termination of the experiment.

The trachea swabs placed in PPLO broth (Difco) with 10 percent horse serum, 10 percent yeast hydrolysate, 1,000 units of penicillin per ml, thallium acetate, 1:4000, and 0.0025 percent phenol red. Transfers were made in 24 hours and three passages were made before plating on PPLO agar (Difco) with 10 percent horse serum and yeast hydrolysate. The colonies were examined for characteristic pathogenic strain morphology.

Individual weights were obtained at 9 weeks and the feed conversion calculated. The degree of respiratory signs were classified weekly as either 0 to 4 in trial 1 and 2, and 0 to 5 in trial 3. The classification recorded as one was a very slight rale and classification 4 or 5 was a marked rale. The 5 classification was used in trial 3 since the respiratory

TABLE I

Signs and lesions of chronic respiratory disease as influenced by chlortetracycline with and without 0.5 per cent terephthalic acid[1]

Level of CTC gm/ton of feed	Degree of rale[2] Trial 1	Degree of rale[2] Trial 2	Mortality % Trial 1	Mortality % Trial 2	Lesions[3] % Trial 1	Lesions[3] % Trial 2	Reactors[4] % Trial 1	Reactors[4] % Trial 2	Isolations[5] % Trial 1	Isolations[5] % Trial 2
					Terephthalic acid - 0.5 percent					
0	3.0	3.3	5.0	4.4	2.5	10.0	95	100	65	55
125	1.1	1.0	2.0	2.2	0	1.1	75	3.0	54	10
250	1.0	1.0	1.5	3.3	0	1.7	19	1.0	73	0
375	0.7	1.2	2.5	3.3	1.0	0.6	2	0	0	0
Control[6]	0.0	0.0	3.0	6.6	0	0.0	0	0	0	0
					No terephthalic acid					
0	2.9	3.1	3.0	4.4	2.5	6.7	92	100	50	77
125	2.4	2.1	2.0	3.3	0.0	2.2	75	89	42	60
250	1.9	1.2	3.0	3.9	3.5	1.7	90	8	42	36
375	1.8	1.3	2.5	2.8	0.0	1.7	59	4	25	42
Control[6]	0.0	0.0	10.0	6.6	0.0	0.0	0	0	0	0

[1] Twenty-five percent of the birds inoculated with *Mycoplasma* culture WVU 1791. Birds vaccinated for bronchitis and Newcastle disease at 10 days of age.

[2] Based on a weekly classification from 1 to 4.

[3] Based on the presence of caseous exudate in the air sacs.

[4] Whole blood test in trial 1 and serum plate test trial 2.

[5] Ten birds cultured for Mycoplasma in trial 1 and 20 birds per pen in trial 2.

[6] These birds were not inoculated, nor vaccinated, and were reared in isolation in batteries.

signs appeared more severe than in trials 1 and 2. The deaths were recorded and the number of birds with air sac lesions was determined either at autopsy or on the dressing line at the time of slaughter.

RESULTS AND DISCUSSION

The birds in all three trials developed marked clinical signs of CRD as shown by the average degree of rale in tables 1 and 3. The rales were more marked in trial 3. This would be expected since all birds were exposed to *Mycoplasma* at day old. Infection was apparently established since a slight rale was noted in a few birds before vaccination for Newcastle disease at two weeks of age. In all trials, a slight decrease in the degree of rales was noted in the birds when they were about 9 weeks of age.

Generally, as the level of the antibiotic increased, the number of birds showing positive agglutination titers (reactors) to the CRD test decreased (tables 1 and 4). The percent isolations did not correlate with the percent reactors

TABLE II

Average weight and feed conversion at 9 weeks as influenced by chlortetracycline (CTC) and terephthalic acid (TPA) in birds exposed to *Mycoplasma*[1]

Level of CTC gm/ton of feed	Average weight (GMS) Trial 1	Trial 2	Feed conversion Trial 1	Trial 2
	Terephthalic acid - 0.5 percent			
0	1294	1331	2.52	2.73
125	1519	1591	2.36	2.64
250	1508	1570	2.42	2.59
375	1525	1551	2.40	2.57
Control[2]	1515	1591	2.32	2.38
	No terephthalic acid			
0	1174	1318	2.59	2.68
125	1509	1506	2.44	2.72
250	1492	1566	2.44	2.61
375	1528	1580	2.34	2.55
Control[2]	1411	1562	2.54	2.59

[1] Twenty-five percent of the birds were inoculated with *Mycoplasma* culture WVU 1791.

[2] These birds were not inoculated nor vaccinated and were reared in isolation in batteries.

(tables 1 and 4). When there were no reactors in a group, no *Mycoplasma* were recovered. This occurred when 250 to 375 gm per ton of CTC with 0.5 percent TPA was fed. In trial 3, table 4, where all the birds were cultured, there were about equal numbers of isolations from the birds showing no reaction to the CRD plate test as compared to those birds positive to the test. It is interesting to note in table 4 the result of the CRD tests during the course of trial 3.

In the uninoculated controls, 81 percent of the birds reacted at 5 days and five percent reacted at 2 weeks after which all tests were negative. In the inoculated groups fed CTC continuously, approximately 55 percent of the birds reacted at 2 weeks. None of these birds reacted at 4 weeks even though they had been vaccinated at 2 weeks of age for Newcastle disease. However, following bronchitis vaccination at four weeks, the reactor rate increased to 40 percent when the birds were tested at 6 weeks. The reactor rate had increased to 53 percent at 9 weeks. When CTC was fed for 2 weeks, the reactor rate was 55, 27, 90, and 100, at four weeks the reactor rate was 45, 30, 73, and 96 when the birds were

TABLE III

Weight, feed conversion, percent mortality, air sac lesions, and degree of rale when birds were given chlortetracycline (CTC 250 gm/ton) and terephthalic acid (TPA 0.5%) for 2, 4, or 9 weeks[1]

Medication	Average weight (Gms)	Feed conversion	Degree of rale[2]	Mortality %	Air sac lesions %
None	1440	2.57	3.3	2.0	1.0
None	1518	2.44	3.3	2.0	1.0
9 Weeks	1616	2.58	2.5	5.0	1.0
9 Weeks	1573	2.48	2.4	1.0	1.0
2 Weeks	1535	2.54	3.5	0.0	2.0
2 Weeks	1565	2.50	3.3	0.0	1.0
4 Weeks	1599	2.48	2.6	0.0	2.0
4 Weeks	1604	2.33	2.5	3.0	2.0
None[3]	1556	2.51	0	8.0	0.0

[1] Birds exposed to *Mycoplasma* culture (WVU 1791) at day-old. Vaccinated for Newcastle disease at 2 weeks and bronchitis at 4 weeks.
[2] Degree of rale based on 0 - 5, 1 slight rale to 5 marked rales.
[3] These birds were not inoculated nor vaccinated and were reared in isolation in batteries.

tested at 2, 4, and 6 and 9 weeks respectively. The infected controls showed a reactor rate of 30 percent at 2 weeks and then 100 percent reacted on each of the other tests. Apparently the bronchitis vaccination at 4 weeks stimulated the CRD process which was not controlled by feeding CTC even though it was given continuously.

One can only theorize with the information available; however, it would appear that the efficacy of the CTC is in proportion to the severity of the disease. Also, the antibiotic acts more effectively in preventing the spread of CRD, therefore, the best use of the CTC would be during periods of stress such as vaccination. The desired level should be no less than 250 gm per ton of feed and under unusual stress it appears that this level should be increased.

If high level antibiotic feeding with TPA is to be used in an eradication program, then higher levels should be used continuously. These data, table 1 and 4, indicate that between 250 and 375 grams per ton of CTC plus 0.5 percent TPA should be given.

The mortality in these experiments was generally less than 5.0 percent and air sac lesions less than 10 percent,

TABLE IV

Percent CRD - reactors and percent isolation of *Mycoplasma* when chlortetracycline (CTC), 250 gm/ton, and terephthalic acid (TPA), 0.5%, were fed for 2, 4, or 9 weeks[1]

Medication	Percent positive CRD reactors					Positive culture %
	5 Days	2 Weeks	4 Weeks	6 Weeks	9 Weeks	
None	81	—	—	—	—	—
None	—	30	100	100	100	35
None	—	30	100	100	100	15
9 Weeks	—	60	0	45	65	34
9 Weeks	—	50	0	35	51	18
2 Weeks	—	65	10	90	100	20
2 Weeks	—	45	45	90	100	50
4 Weeks	—	50	30	60	96	40
4 Weeks	—	40	30	85	96	45
None[2]	—	5	0	0	0	0

[1] All birds exposed to *Mycoplasma* culture WVU 1791 at day old, vaccinated for Newcastle disease at 2 weeks and bronchitis at 4 weeks (100 birds per pen).

[2] These birds were not inoculated nor vaccinated and were reared in isolation in batteries.

table 1 and 4. The high mortality in the control groups reared in batteries resulted in most cases from visceral gout which occurred during the first 10 days of brooding. The air sac lesions in trial 1 were based on autopsy of the dead birds and examination of those condemned at slaughter. In trial 2 and 3, the birds were observed on the dressing line for the presence of air sac lesions. This may account for the increase in the number of birds with air sac lesions in trial 2 but does not explain the small number of birds with air sac lesions in trial 3. These birds were also observed at time of slaughter. Even at the CTC level of 375 gm per ton of feed plus TPA, 1 per cent or less of the birds showed lesions of air sac infection. This may indicate that under field conditions such levels of antibiotics may not control the air sac infection completely, but would probably reduce its incidence.

There was a marked difference in final weight between the birds fed CTC and the infected birds that received no antibiotic. The feed conversion difference was not so marked but was generally higher in those infected birds fed no CTC (tables 2 and 3). These experiments did not show the most economical antibiotic level for maintaining growth, however, the continuous medication was only slightly better than feeding the antibiotics for a 2 or 4 week period.

Based on degree of rale, mortality, air sac lesions, recovery of mycoplasma and reactor rate, the 125 gram concentration of CTC with TPA compared favorably with the 375 gram level CTC without TPA. It is, therefore, within reason to assume that CTC is potentiated by TPA approximately 2 to 3 times. Since regulations are such that TPA cannot be used for broilers at this time, the use of low calcium diets should be investigated. The broiler grower is interested in a level of CTC that would reduce mortality, provide satisfactory weight gain and feed conversion and reduce condemnations. This level was not determined by these studies.

Further investigations directed toward the rearing of birds free of CRD by using high levels of antibiotic plus TPA during the growing period should be investigated. The level

of CTC to use would appear to be in the neighborhood of 300 to 400 grams CTC per ton of feed.

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SUMMARY

Chlortetracycline, 125, 250, or 375 grams per ton of feed, with and without 0.5 percent terephthalic acid was effective in preventing the retardation of weight as a result of chronic respiratory disease. Chlortetracycline, 375 grams per ton of feed, with 0.5 percent terephthalic acid prevented the development of CRD. It was estimated that terephthalic acid potentiated CTC from 2 to 3 times.

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MORPHOLOGICAL STUDIES OF AVIAN PPLO IN SEMI-SOLID MEDIA*†

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THE use of a semi-solid medium for the cultivation of PPLO (pleuropneumonia-like) organisms from cases of nongonorrheal urethritis has been reported by Beveridge.[1] Edward[2] has reported semi-solid agar to be useful in characterizing strains of PPLO. Both Beveridge and Edward utilized semi-solid agar to facilitate the isolation of PPLO. In addition, Edward reported the growth characteristics of several strains of PPLO with respect to the location of growth in relation to the surface of the medium. He also described the appearance of the growth, classifying it as smooth, granular or fluffy. Apparently this description of appearance referred to the gross cultural appearance. However, it was not apparent whether petri plates or test tubes were used in this experiment. Edward also reported that PPLO should be cultivated using "the minimum concentration of agar . . . , since the growth of some strains is impaired by too stiff a gel".

Since a description of single colonies of pathogenic avian PPLO growing in a semi-solid medium has not been described in the literature and since several interesting and useful characteristics exhibited by these organisms may be observed by this technique, it appears desirable to report the findings of such a study at this time.

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MATERIALS AND METHODS

Forty-eight hour broth* cultures of pathogenic avian PPLO strains classified as *Mycoplasma gallisepticum* and designated W (Winchester), VB (Winchester strain as carried at Cornell), 293 (standard Cornell pathogenic strain), and S-6 (standard California pathogenic strain) were diluted logarithmically to the base 10 through tubes of liquefied 0.42 percent (semi-solid) agar† (empirically selected as the most satisfactory agar concentration for these experiments) held at 45 C. Immediately after inoculation, the tubes were removed from the 45 C water bath to limit the effect of heat on the inocula. They were subsequently incubated at 37 C for one or more weeks or until individual colony morphology could be clearly observed. Comparable glucose broth§ dilutions of each culture were also made, and the effect of heat (45 C) on the cultures was determined.

To test the capacity of the semi-solid medium to support growth and to maintain culture viability for relatively long periods of time, tubes of the medium were inoculated with the previously named cultures by placing one drop of a 48 hour culture on the surface of the medium and stabbing through this inoculum with a sterile bacteriological loop to the bottom of the tube. These cultures were incubated for one week at 37C., stored at room temperature, and subcultured weekly with a bacteriological loop to tubes of semi-solid agar and to tubes of glucose broth.

RESULTS

PPLO growing in tubes of 0.42 percent semi-solid agar

* Difco PPLO Broth w/o CV, 1/4000 parts thallium acetate, 1 per cent Difco PPLO Serum Fraction, adjusted to a final pH of 7.8.

† 7 parts Difco PPLO Broth w/o CV, 3 parts PPLO agar, 1/4000 parts thallium acetate, 1 per cent Difco PPLO Serum Fraction, adjusted to a final pH of 7.8.

§ As above † but without agar and containing 0.25 per cent dextrose and phenol red.

produced areas of growth ranging from narrow bands near the surface of the medium when heavily inoculated to well isolated colonies growing throughout the upper one-half to two-thirds of the medium when lightly inoculated.

Figure 1 illustrates what may generally be observed when *Mycoplasma gallisepticum* strains are studied in semi-solid agar. From left to right, the tubes pictured received inocula diluted to 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. It can be observed that heavy growth occurred in a zone just below the surface

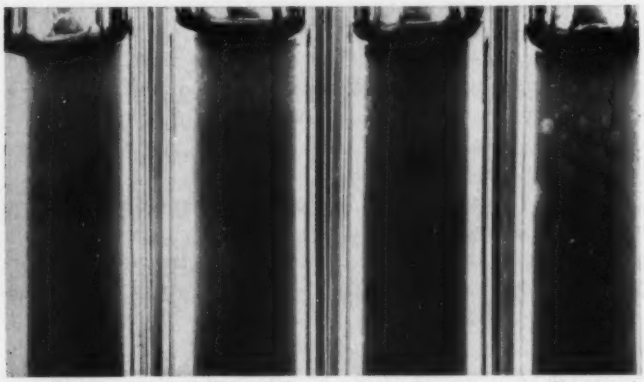

Fig. 1. From left to right the test tubes (2 cm. in diameter) contain semi-solid agar with growth initiated from 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions of 48 hour broth cultures of *Mycoplasma gallisepticum*, W strain.

The extreme variation in colony size is apparent in the tube to the right. The relationship of inoculum concentration to the level of growth and to colony size is also apparent.

of the medium in the tube which received the most concentrated inoculum. In the tubes which received more dilute inocula, individual colonies became apparent and grew throughout a wider zone. In the tube which received the most dilute inoculum, the colonies are quite distinctly separate, are no longer of uniform size and grow throughout the widest zone. The size of individual colonies is apparently limited by available space, the length of incubation time, and the redox potential of the medium, under the conditions described.

Strains S-6, 293, and several other W strains which were

similarly observed produced growth virtually identical to that pictured in figure 1. However, the W strain as carried at Cornell University produced smaller colonies of uniform size.

Comparative viability titrations in semi-solid medium and in glucose broth were not always in agreement. The broth usually was the more sensitive of the two media. Heat sensitivities run in broth indicate that the phenomenon apparently is not due to heat since the ability of dilute inocula to initiate growth was not reduced by holding inoculated tubes of media at 45 C for as long as 40 minutes.

PPLO stored in semi-solid media at room temperature and transferred with a bacteriological loop remained viable for as long as four weeks.

DISCUSSION

Semi-solid agar, as herein described, appears to be a suitable medium for observing PPLO colony morphology. The observation that well separated PPLO colonies growing in semi-solid media tend to grow more prolifically near the center of the test tube, as opposed to growing near the surface or near the bottom, indicates that growth of these organisms is favored by a somewhat reduced oxygen tension.

The variation observed in colony morphology could be an indication that the cultures observed normally dissociate into both large and small colonies or that the strains are not "pure" cultures. The fact that the "VB" strain is a purified "W' strain and exhibits but a single colony type seems to favor the latter hypothesis. This phenomenon should be investigated further since it might be related in some way to other characteristics of the organisms such as serology, pathogenicity or biochemical activity.

Semi-solid agar stab culturing may prove to be a superior method for the maintenance of stock PPLO cultures, since PPLO are more protected from desiccation in semi-solid agar than when propagated on the surface of agar plates and multiply less prolifically in it than they do in liquid media.

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SUMMARY

Mycoplasma gallisepticum strains designated W, S-6, 293 and VB were serially diluted and inoculated into tubes of 0.42 percent semi-solid agar. All strains grew well in this medium and produced well isolated colonies suitable for comparative morphological studies. Colonies produced by all strains were virtually identical in appearance except those produced by the VB strain which were markedly smaller and more uniform in size.

All strains grew more prolifically and in a narrow band near the surface of heavily inoculated tubes of media, and nearer the center and throughout a much wider zone in lightly inoculated tubes of media. Under the conditions of the experiment, the size of individual colonies seems to be limited only by space and the length of time of the incubation period.

Semi-solid agar stab-culturing appears to be a suitable or possibly superior method for the maintenance of PPLO stock cultures.

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THE EFFECTS OF ANTIBIOTICS ON EXPERIMENTAL CHRONIC RESPIRATORY DISEASE IN CHICKENS*

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VARIOUS chemotherapeutic agents have been investigated relative to their influence on experimental and natural chronic respiratory disease (CRD) infection. Results obtained from *in vitro, in ovo,* and *in vivo* studies have been extremely variable. Olesiuk *et al.*[2] have recently reviewed the literature on this subject. Yamamoto and Adler[6] and Hamdy *et al.*[1] reported chlortetracycline (CTC) and oxytetracycline (OTC) among the most active antibiotics in their *in vitro* studies with CRD.

Recent research[3,4,5] on the potentiation of antibiotics by certain compounds has aroused considerable interest. Terephthalic acid (TA) has been one of the most promising of these compounds. Serum levels of orally administered CTC and OTC have been enhanced by simultaneous feeding of TA.

In these studies, CTC and OTC (plus TA) were evaluated for their ability to maintain or to stimulate weight gains, to reduce, suppress, or inhibit the CRD syndrome when administered in the feed to chickens experimentally infected with noncomplicated CRD.

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EXPERIMENTAL PROCEDURE

In a series of six experiments, CTC and OTC (plus TA) were investigated relative to their influence on experimental CRD infection. The chickens (5 to 9½ weeks of age) used in this investigation were obtained from CRD-free stock of the following breeds: White Leghorn (WL), White Rock (WR), and Sex-Link (SL). The chickens were reared at the laboratory under strict isolation and only in one experiment (experiment 3) the birds were exposed to vaccination for Newcastle disease and infectious bronchitis. The birds were maintained on a bagasse litter in an experimental poultry house containing six pens that simulated commercial field conditions.

The CRD inoculum was strain Hy (S_6 type) of *Mycoplasma gallisepticum* that was isolated from a Massachusetts flock naturally infected with CRD. The virulence of this strain was maintained by continuous bird passage. Bird-passed material was used in five trials and embryonic fluids in one trial. The embryo infective $dose_{50}$ ($e.i.d._{50}$) titer of the inocula was determined in experiments 4, 5, and 6. Sensitivity studies with strain Hy (5th embryo passage) revealed high *in vitro* activity of OTC, the lethal dose being 1 microgram/ml of medium. The birds were infected by inoculation of 0.5 ml of the inoculum into the trachea.

The experiments were varied in regard to the drug levels, time and period of treatment, and overall duration. At weekly intervals, clinical observations were made and blood samples were collected for CRD serological tests with the rapid-serum-plate test. The Adler S_6 strain was used for preparation of the antigen by this laboratory. The results of terminal clinical observations and serologic tests are presented in the succeeding tables. The birds were weighed individually at the onset and termination of each experiment.

At the time of necrospy, the gross pathological changes in the larynx, trachea, post thoracic, lesser, and greater abdominal air sacs were scored as a slight or marked reaction. The trachea and nasal passages from a portion of the birds

were cultured for PPLO re-isolation. The first three series of experiments were conducted in 1958 while the latter three were conducted in 1959.

Experiment 1. This experiment was designed to evaluate the prophylactic effect of CTC at 200 gm/ton of feed on experimental CRD. A total of 358, 9-week-old WR chickens were allotted on an equal sex basis into five pens of 45 to 90 birds each. The CRD inoculum was the 5th bird passage of strain Hy. Noninoculated birds were maintained in one pen as controls and additional noninoculated birds were maintained in two of the inoculated pens as contact controls. Two groups were placed on the antibiotic regimen at the time of CRD exposure and continued on this ration throughout the 4-week duration of the experiment.

Experiment 2. This was an evaluation of the prophylactic effect of CTC on CRD at 200 gm/ton of feed. Three hundred and seventy-eight, 6½- and 7-week-old WR chickens were divided on an equal sex basis into four pens of 30 to 100 birds each. The 8th bird passage of strain Hy was the CRD inoculum. Noninfected birds were maintained in one pen as controls and additional controls were maintained in two of the inoculated groups as contact controls. The chickens in two groups were placed on the antibiotic regimen at the time of CRD exposure and continued on this ration throughout the 4-week duration of the experiment.

Experiment 3. This experiment was designed to determine the response or benefit obtained by the use of CTC therapy at 200 gm/ton in experimentally inoculated CRD birds that were subsequently stressed by Newcastle and bronchitis vaccination. A total of 262, 9½-week-old WL males were distributed into four pens of 40 to 91 birds each. Designated lots were infected with the 10th bird passage of strain Hy. Noninfected controls were added to the inoculated groups at the time of CRD exposure and 2 weeks post-CRD inoculation. At 2 weeks post-CRD inoculation the birds in all four groups were exposed by aerosol to a mild strain of infectious bronchitis virus and B_1 Newcastle virus. At this time two

groups of birds were placed on the antibiotic regimen and continued on this ration until the termination of the experiment at 8 weeks.

Experiment 4. This was a determination of the influence of OTC on CRD at 200 gm/ton of feed plus 0.25 percent and 0.5 percent levels of TA. Three hundred and ninety-two, 8-week-old SL chickens were allotted on an equal sex basis into six pens of 61 to 67 birds each. The CRD inoculum was the 5th embryo passage of strain Hy and had an e.i.d.$_{50}$ titer of 10^{-7} in 8-day embryos. Noninfected controls were maintained in two pens and at 11 days post-CRD exposure noninfected controls were placed in the inoculated groups to serve as contact controls. Four groups of birds at 12 days post-CRD inoculation were placed on the antibiotic regimen and continued on this ration until the termination of the experiment at 6 weeks.

Experiment 5. The therapeutic efficacy on experimental CRD of OTC at 200 gm/ton of feed plus 0.25 percent and 0.5 percent levels of TA was studied in this experiment. Four hundred and sixty-two, 6½-week-old WR chickens were distributed on an equal sex basis into six groups of 63 to 105 birds each. The CRD inoculum was the 12th bird passage of strain Hy and had an e.i.d.$_{50}$ titer of 10^{-4} in 7-day embryos. Noninfected contact controls were maintained in two pens, and at 11 days postinoculation, additional susceptible birds (contact controls) were placed in these pens. Birds in two pens were maintained as noninoculated controls. Four pens were placed on the antibiotic regimen at 11 days postinoculation and continued on this feed until the termination of the experiment at 6 weeks.

Experiment 6. In this experiment the prophylactic effect of OTC on CRD at 200 gm/ton of feed plus 0.5 percent and 1 percent levels of TA was determined. Four hundred and eighty-nine, 5-week-old WR chicks were allotted on an equal sex basis into six groups of 66 to 110 birds each. Designated lots were infected with the 13th bird passage of strain Hy that had an e.i.d$_{50}$ titer of 10^{-4} in 7-day embryos. Noninfected contact controls were maintained in two pens. Four

pens were placed on the antibiotic regimen at the time of CRD inoculation and continued on this feed throughout the 4-week duration of the experiment.

RESULTS

The results of experiment 1, a determination of the prophylactic efficacy of CTC on experimental CRD, are given in table 1. As indicated previously the birds in pens A and E were inoculated with CRD, a portion of the birds in pens B and D were inoculated, and the birds in pen C were maintained as noninoculated controls.

The infected birds in groups A and B showed the lowest weight gains while the birds in control group C and the controls of group B showed the highest gains. The weight gains in the two inoculated-treated pens, D and E, were slightly lower than those obtained in control pen C.

Clinical signs of CRD were observed in 37 percent of the birds in group A and in 27 percent of the inoculated birds in group B at the termination of the experiment. A small percentage of the birds in groups D and E showed respiratory signs while the group C birds were negative.

The inoculated birds of groups A and B showed extensive and severe gross pathological changes in the larynx, trachea, and air sacs. There was only slight evidence of CRD transmission among the contact controls of group B. The lesions in the birds of groups D and E were not as extensive or severe as those observed in groups A and B.

A higher percentage of the birds were positive serologically in the inoculated-nontreated pens than in the inoculated treated pens. The antibiotic may have inhibited the development of the CRD syndrome in some of these birds.

The trachea and nasal passages of birds selected at random from the various groups were cultured for PPLO. The number of positive isolations over the number of birds cultured are listed by groups: A - 3/6, B (inoculated) - 1/2, B (contact controls) - 1/5, C - 0/5, D (contact controls) 0/5, and E - 5/5.

The results of experiment 2 are presented in table 1. The

TABLE I

Influence of chlortetracycline on experimental CRD

Exp.	Groups	No. birds	Medication (ton) CTC (gms)	Average weight gained (percent)	Percentage birds positive							Percentage air sacs positive [2]	
						L & T		Air sacs [1]		Total			
					Signs	S	M	1-3	4-6	lesions	Serology	S	M
	A	89	0	42	37	12	54	22	38	66	69	21	18
	B	15	0	45	27	20	40	47	7	60	60	11	11
1.		30*	0	56	3	7	3	0	0	10	3	0	0
	C[3]	45	0	53	0	0	0	0	0	0	0	0	0
	D	44	200†	51	5	25	9	39	0	41	41	9	3
		46*	200†	52	0	0	0	0	0	0	2	0	0
	E	89	200†	51	6	28	22	51	4	56	54	16	4
	F	50	200†	102	18	44	30	14	40	78	76	29	12
		50*	200†	113	0	20	2	2	0	24	4	0	0
	G	99	200†	101	17	35	35	15	49	74	76	43	9
2.	H	100	0	94	43	23	59	17	54	88	88	30	26
		49*	0	104	6	6	6	4	2	12	20	1	2
	I[3]	30	0	105	0	0	0	0	0	0	0	0	0
	J	49	0	90	2	37	6	53	12	73	100	26	0
		22*	0	100	41	23	73	55	18	100	100	29	4
		20**	0	94	25	50	50	55	15	100	100	26	4
3.	K	48	200‡	89	0	8	0	38	0	42	98	11	0
		21*	200‡	106	5	38	10	24	0	57	67	5	2
		22**	200‡	100	14	23	27	27	0	64	55	6	2
	L[3]	40	0	109	0	0	0	0	0	0	0	0	0
	M[3]	40	200	106	0	0	0	0	0	0	0	0	0

† Medication period: 1 to 28 days post-CRD inoculation.
‡ Medication period: 14 to 56 days post-CRD inoculation.
[1] Air sacs: Right and left post thoracic, lesser, and greater abdominals.
1-3, 4-6 = Number of air sacs.
[2] Six air sacs scored for each bird.
[3] Noninoculated controls.
* Contact controls placed at onset. ** Contact controls placed at 14 days post-CRD exposure.
L & T = Larynx and trachea. S = Slight. M = Marked.

birds in group G were inoculated with the CRD strain, a portion of the birds in groups F and H were inoculated, and group I was maintained as the noninoculated group.

The lowest weight gains were obtained in the inoculated-nontreated birds while the highest weight gains were obtained in the noninfected controls. The contact controls of group F showed an 8 percent gain over group I and a 9 percent gain over the group H contact control birds.

The group H birds revealed a higher percentage of signs than did the inoculated-treated birds of groups F and G. There was a definite suppression of respiratory signs among the birds that were given the antibiotic.

The gross lesions observed in the birds of group H were more numerous and extensive or severe than in the inoculated-treated birds of groups F and G. A higher percentage of gross lesions in the upper respiratory tract was observed among the contact controls of group F than in the contact controls of group H. It should be noted, however, that the lesions in the contact controls of group F were very slight.

Of the CRD-exposed groups, group H had the highest percentage of birds positive serologically for CRD.

Cultures for PPLO were not made at the termination of this experiment.

Experiment 3, summarized in table 1, was a determination of the therapeutic efficacy of CTC on experimental CRD in chickens stressed by vaccination with Newcastle and infectious bronchitis viruses at 2 weeks post-CRD exposure. A portion of the birds in groups J and K were inoculated with the CRD strain while groups J and K were inoculated with noninoculated controls.

The highest weight gains were obtained in the noninoculated birds of groups L and M. The weight gains in groups J and K were 90 percent and 89 percent, respectively, for the inoculated birds. The weight gains of the contact controls of group K were higher than those of group J.

Respiratory signs were most numerous in the nontreated group J birds.

* Contact controls placed at onset.
L & T = Larynx and trachea. S = Slight. M = Marked.

Lesions were more extensive and severe in the inoculated and contact control birds in group J than in the birds in group K. Group K was treated with CTC at 2 weeks post-CRD exposure.

It should be noted that all the birds in group J were positive serologically while in group K, the treated group, there were negative birds. The antibiotic apparently inhibited the CRD syndrome among those birds.

Isolations of PPLO were made from all CRD-exposed groups. The number of PPLO isolations over the number of birds cultured in groups J and K are listed: group J, inoculated birds - 2/5, initial contact controls - 2/4, 2-week contact controls - 2/5; group K, inoculated birds - 3/5, initial contact controls - 2/6, 2-week contact controls - 3/5. The 16 birds that were cultured from groups L and M were negative for PPLO.

The results of experiment 4, a study of the therapeutic efficacy of OTC supplemented with graded levels of TA on experimental CRD, are presented in table 2. Groups A-1, A-2, B-1, and B-2 were the CRD-inoculated groups while groups C and D were the noninoculated groups.

The infected-control group, B-1, showed the lowest weight gains of all groups while the treated-control group C and the treated-contact controls of group B-2 revealed the highest weight gains. The weight gains of group B-2, the group given OTC alone in the feed, revealed higher weight gains than groups A-1 or A-2, the antibiotic plus TA groups.

A small percentage of the birds in groups A-1, A-2, and B-1 revealed respiratory signs. However, the group A-1 birds revealed only slight clinical signs of CRD.

The gross pathology of group B-1 was more severe and extensive than in the other groups of birds. The contact controls of this group showed a higher percentage of lesions than did the controls in the remaining groups. The results of groups A-1, A-2, and B-2 were very similar and difficult to evaluate on a basis of gross pathology.

The inoculated birds in all groups were positive serologi-

cally. The majority of the contact control birds in group B-1 were positive serologically while only a few control birds were positive in the remaining inoculated groups.

Six inoculated birds were cultured for PPLO from each of the 4 CRD-exposed pens. The number of isolations are listed by pens: A-1 = 4, A-2 = 3, B-1 = 1, and B-2 = 1. Two contact controls were cultured from each of these pens and eight birds from pens C and D, respectively. The controls were all negative on culture.

The results of experiment 5, an evaluation of the therapeutic effect of OTC supplemented with TA on experimental CRD, are listed in table 2. The birds in groups A-2 and B-2 were inoculated with the CRD strain, the majority of the birds in groups A-1 and B-1 were inoculated, and groups C and D were maintained as controls.

The lowest weight gains were obtained in group B-1, the nontreated group, and the highest weight gains were obtained in one group of contact controls of group A and in the control groups C and D.

The groups are difficult to evaluate on a basis of respiratory signs and lesions; however, the treated groups appeared to be more severely involved than the nontreated groups. The contact controls of group B-1 showed a higher percentage of signs and lesions than did the A-1 group.

The inoculated birds in all groups were positive serologically at the termination of the experiment. However, a portion of the controls in group A-1 remained serologically negative.

Six inoculated birds were cultured from each of the exposed pens. Single isolations of PPLO were made from pens A-2 and B-1, while the results were negative for pens A-1 and B-2. Three birds were cultured from each lot of contact controls of groups A-1 and B-1. In group A-1 only a single isolation was made from the controls introduced at the onset of the experiment. In group B-1, PPLO was isolated from one of the initial contact controls and three isolations were made from the controls introduced at 11 days post-CRD exposure.

Data and results of experiment 6 are presented in table 2.

TABLE II

Influence of oxytetracycline supplemented with terephthalic acid on experimental CRD

Exp.	Groups	No. birds	Medication OTC Gm/ton	Medication TA Percent	Average weight gained (percent)	Percentage birds positive: Signs	L & T S	L & T M	Air sacs 1 1-3	Air sacs 1 4-6	Total lesions	Serology	Percentage air sacs positive 2: S	Percentage air sacs positive 2: M
4.	A-1	59	200†	.5	94	5	58	0	39	34	95	100	38	2
		8**	200†	.5	104	0	13	0	13	0	25	25	2	0
	A-2	59	200†	.25	95	3	68	3	39	32	92	100	33	5
		8**	200†	.25	98	13	13	0	0	0	13	0	0	0
	B-1	53	0	0	88	9	65	25	32	47	100	100	35	14
		8**	0	0	101	13	50	13	0	0	63	75	0	0
	B-2	60	200†	0	97	0	73	0	32	42	88	100	37	11
		5**	200†	0	110	0	0	0	0	0	0	20	0	0
	C[3]	66	200†	0	105	0	0	0	0	0	0	0	0	0
	D[3]	66	0	0	100	0	0	0	0	0	0	0	0	0
5.	A-1	68	200‡	.5	108	1	60	1	43	51	99	100	55	2
		17*	200‡	.5	121	0	29	6	0	6	35	71	4	1
		20**	200‡	.5	106	0	5	5	15	0	15	40	4	2
	A-2	65	200‡	.25	110	5	65	3	46	35	91	100	43	1
	B-1	66	0	0	104	0	64	5	55	35	97	97	46	4
		15*	0	0	103	20	40	7	47	7	73	100	17	3
		20**	0	0	104	5	40	25	20	5	65	95	12	2
	B-2	64	200‡	0	108	0	59	2	31	59	98	100	63	3
	C[3]	63	200‡	0	121	0	0	0	0	0	0	0	0	0
	D[3]	64	0	0	119	0	0	0	0	0	0	0	0	0

TABLE II *(Continued)*

	A-1	87	200§	1.0	122	25	56	33	38	61	99	100	61	3
		21*	200§	1.0	131	0	10	0	0	0	10	5	0	0
	A-2	68	200§	.5	121	31	57	22	22	75	99	99	67	6
	B-1	89	0	0	104	55	34	65	17	82	100	100	66	10
		21*	0	0	127	10	0	14	5	10	14	48	5	6
6.	B-2	67	200§	0	111	30	40	54	19	81	100	100	69	11
	C[3]	70	200§	1.0	135	0	0	0	0	0	0	0	0	0
	D[3]	66	0	0	124	0	0	0	0	0	0	0	0	0

† Medication period: 12 to 42 days postinoculation.

‡ Medication period: 11 to 42 days postinoculation.

§ Medication period: 1 to 28 days postinoculation.

[1] Air sacs: Right and left post thoracic, lesser, and greater abdominals. 1-3, 4-6 = Number of air sacs.

[2] Six air sacs scored for each bird.

[3] Noninoculated controls.

* Contact controls placed at onset.

** Contact controls placed at 11 days postinoculation.

L & T = Larynx and trachea. S = Slight. M = Marked.

This experiment evaluated the prophylactic effect of OTC plus TA on experimental CRD. The birds were necropsied at 4 weeks post-CRD inoculation. The majority of the birds in groups A-1 and B-1, and all the birds in groups A-2 and B-2 were inoculated with the CRD strain. Groups C and D were maintained as noninoculated controls.

The highest weight gains were obtained in the treated-control group C. The poorest weight gains were obtained in the inoculated birds of groups B-1 and B-2. The weight gains of A-1 and A-2 were similar to those made by control group D.

The nontreated inoculated group B-1 revealed the highest incidence of signs and lesions. The group treated with OTC alone showed a higher incidence of signs and lesions than did the groups treated with OTC plus TA.

The inoculated birds were positive serologically at the termination of the experiment. One contact control in group A-1 and 10 contact controls in group B-1 were positive serologically.

Cultures were made from birds selected at random from the various groups. No isolations were made from the controls of groups C and D. In group A-1, two PPLO isolations were made; one from the four contact controls cultured and one from the eight inoculated birds cultured. In group A-2, three PPLO isolations were made from the seven birds cultured and six isolations from eight birds cultured in group B-2. In group B-1, six PPLO isolations were made; four from the eight inoculated birds cultured and two from the five contact controls cultured.

DISCUSSION

A series of six experiments, in which the prophylactic and therapeutic effect of CTC and OTC supplemented with graded levels of TA on experimental CRD in chickens, 5 to 9½ weeks of age, are reported. The following criteria for drug evaluation were used: weight gains, clinical signs, gross pathological changes, serology, and cultural tests.

In evaluating the results obtained in this investigation

relative to the influence of CTC and OTC (plus TA) on noncomplicated CRD, it may be stated that these products exerted only slight inhibition on the course and severity of CRD. These products stimulated weight gains in CRD-infected birds and also in control birds. It was observed in only one experiment that TA supplementation of OTC appreciably increased the effectiveness of OTC in stimulating weight gains in CRD-infected birds.

The general experimental design has an important influence on the results obtained. The time and duration of medication and the duration of the experiment may influence the results obtained. In experiments 3, 4, and 5, that were of 6 to 8-weeks duration, the disease syndrome was not as severe at the termination as was observed in experiments 1, 2, and 6, that were terminated at 4 weeks. The age, breed, strain, and sex of birds may have some influence on the results obtained.

The results obtained from the cultural tests were interesting. In experiments 1, 3, and 6, PPLO isolations were readily made. In experiment 4, only a single isolation was made on culture from the inoculated controls. Fifty percent of the cultures taken from the birds that were treated with OTC supplemented with TA yielded PPLO. In experiment 5, only a few isolations were made from the various inoculated groups that were cultured. Apparently, it is difficult to recover PPLO from treated as well as nontreated birds after a certain time interval.

Another important factor that should be considered is the CRD inoculum. Virulence and potency of the inoculum may influence the pattern of results obtained. CRD infections that are complicated by viruses or other bacteria produce a pathological condition in birds that may respond differently to chemotherapy than noncomplicated CRD.

These results confirm the earlier findings of the authors and other investigators. However, it appears desirable that prophylactic and therapeutic determinations be made on CRD complicated with viruses and other bacteria, especially *E. coli*.

SUMMARY

The weight gains obtained in the CRD-inoculated birds treated either with chlortetracycline or oxytetracycline were higher in all but one group than those obtained in the non-treated-inoculated birds. The groups treated with oxytetracycline plus terephthalic acid exhibited in some cases slightly higher weight gains than those treated with the oxytetracycline alone. The noninoculated groups exhibited the highest weight gains. Chlortetracycline and oxytetracycline exerted only slight inhibition of the signs and lesions of the disease.

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AN ANAEROBE, THE CAUSE OF ULCERATIVE ENTERITIS, "QUAIL DISEASE"

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"QUAIL disease" has been reported in different species; western quail, grouse[14,15], wild and domestic turkeys[23], chukar partridge[9], pigeons[8], and chickens[6]. Several workers have reported the isolation of bacteria that would cause ulcerative enteritis when fed to quail. Morley[16] in 1936 isolated a Gram positive, aerobic, non-motile, pleomorphic rod. The majority of strains soon lost their virulence. Bass[2] in 1939 isolated seven times pure cultures of a Gram negative anaerobe which, when fed to quail, killed them with ulcerative enteritis. Durant and Doll[5] in 1941 tested 70 bacterial cultures they had isolated from quail by aerobic and anaerobic culture. None of these cultures reproduced the disease on feeding. On the basis of histological studies and the character of the disease, they concluded the disease was probably of bacterial origin.

This report describes the isolation of a Gram positive, anaerobic, spore-forming bacillus from the liver and blood of quail with intestinal and liver lesions of ulcerative enteritis and from the liver of a chicken affected with ulcerative enteritis.

EXPERIMENTAL

The first case in chickens occurred in a flock of 2,700 6-week-old White Leghorns. Prior to mortality in the flock,

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the heat had been reduced in the brooder house. Ten birds had died in 3 days. Five dead birds submitted for examination had lesions in the livers and intestines. The liver lesions were either small, sharply demarcated, necrotic, yellow foci, fig. 3 or large, poorly delineated, light yellow areas on the border of the liver. The intestinal lesions were yellow, diphtheritic, necrotic ulcerations in the mucosal surface of the ceca and lower half of the intestines (figure 1, 2). Some of the ulcerations had perforated the intestinal wall causing peritonitis.

Transmission of the disease from the first case in chickens was done by feeding an intestinal suspension from one chicken with lesions of ulcerative enteritis to a quail which died within 40 hours. Intestinal material from this quail was fed to a second quail. A blood sample was taken from the wing vein 2 days later, shortly before the death of this quail. The blood was inoculated into the yolk sac of five 6-day-old embryos, all of which died on the second day. A portion of the blood inoculated on blood agar failed to yield any growth. In the yolk from the dead embryos, a bacillus was found on smears stained with basic fuchsin. Two other isolations in embryos were made from the livers of quail that died after being fed different passages of the original yolk isolate.

The second case in chickens occurred in a flock of 1,000 8-week-old White Leghorns. Sulfonamid medication had just been completed following an outbreak of coccidiosis. Six dead birds were submitted for examination. These birds had ulcerations in the ceca and intestine and necrotic foci in the liver. Small gray foci in the liver were crushed between two slides. Examination of the smear stained with Gram revealed Gram positive bacilli and unstained bodies resembling spores occurring free and in the terminal portion of the bacilli. A fourth isolation was made from the liver of a chicken in the second outbreak. A small piece of the chicken liver was triturated in a Ten Broeck grinder with tryptone broth. One portion of the suspension was heated in a water bath for 15 minutes at 60 C before inoculation into three embryos and the other, unheated, was inoculated directly into three embryos. All of the embryos

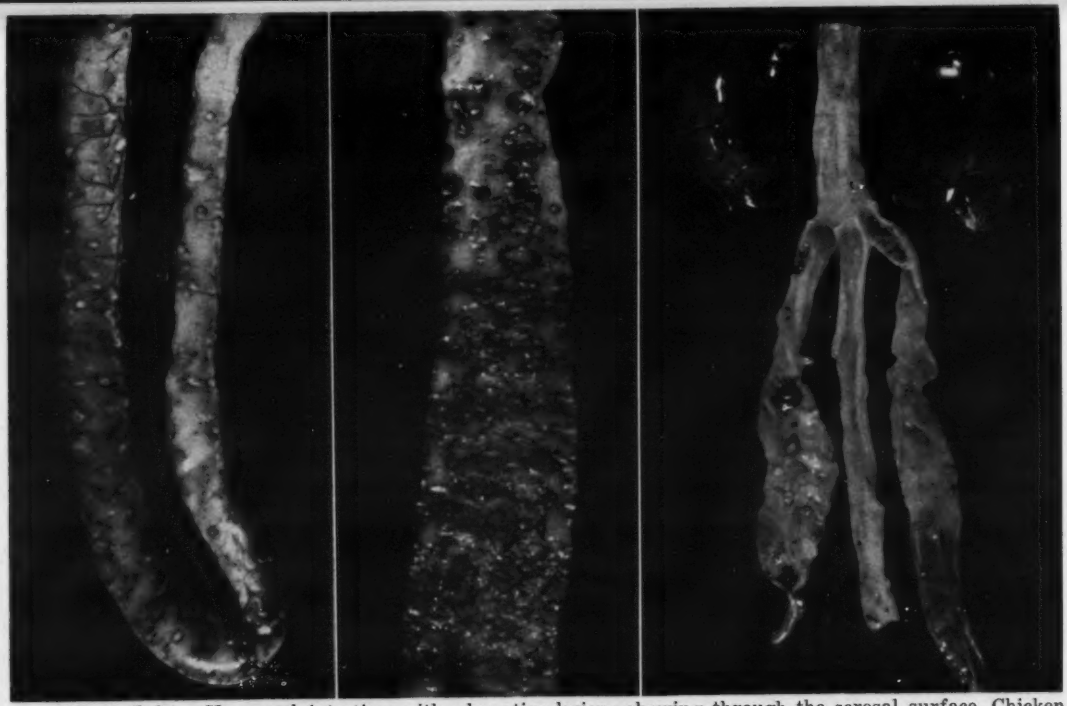

Fig. 1 (left). Unopened intestine with ulcerative lesions showing through the serosal surface. Chicken age 6 weeks.

Fig. 2 (center). Mucosal surface of intestine shown in figure 1. Note necrotic diphtheritic, mucosal lining and yellow ulcerations with a hemorrhagic border.

Fig. 3 (right). Liver with necrotic foci. Ulcerations in mucosal surface of ceca. Chicken, age 7 weeks.

inoculated with the unheated sample died in 48 hours. One embryo inoculated with the heated sample died in 48 hours and the remaining two embryos died in 72 hours. From the yolks of both sets of embryos an anaerobic bacillus was demonstrated with a morphology and spores similar to the bacillus isolated in embryos from quail. A fifth isolation in embryos was made from the liver of a quail that died 39 hours after inoculation with an intestinal suspension from a chicken in the second outbreak dead with ulcerative enteritis.

Cultural characteristics. The bacillus is Gram positive in young cultures and yolk smears from dead embryos. Organisms in old cultures, particularly those grown in artificial media, are decolorized more readily than those from fresh cultures. The bacillus is 3 to 4 microns long and occurs singly as a straight rod or a slightly curved rod with rounded ends. Occasionally, organisms undergoing binary fission are found that are joined at the ends by a fine strand. In embryo cultures, subterminal spores are readily formed (figure 4). The spores occupy the terminal third of the cell and have a cylindrical form with rounded ends. When stained with basic fuchsin, the bacillus stains red and the spore is clear. The spores stain green with the Wirtz spore stain. Growth has been obtained with difficulty on the surface of blood agar plates incubated under anaerobic conditions. Repeated attempts failed to establish growth of the organism in glucose agar shakes. The organism could not be grown aerobically. A yolk suspension of the bacillus heated for 2½ hours at 60 C in a water bath was still lethal for embryos. *In ovo* studies indicated the organism is sensitive to streptomycin at levels of 10 mg per ml of inoculum and to penicillin at levels of 10,000 units per ml of inoculum.

Pathogenicity studies. Limited attempts to infect pheasants, poults, and chicks by feeding yolk cultures have been unsuccessful. Yolk suspensions were without effect when inoculated intraperitoneally into 3-week-old mice. The organism was not pathogenic for guinea pigs when injected into thigh muscles using the calcium chloride technique described by Smith[24]. Infectious yolk when passed through a Seitz filter had no effect when inoculated into embryos.

Quail inoculated orally with one ml of yolk from first, second, and third embryo passages of the organism died in 2 to 3 days. Liver lesions varied from a light yellow mottling to a massive necrosis involving both lobes. Congestion and hemorrhages were found in the spleens. Intestinal ulcers were variable both in extent and number. Ulcers were found throughout the intestine and ceca. In some cases, ulcers were small with a yellow necrotic center surrounded by a hemorrhagic border. Other ulcerations were relatively large, yellow, necrotic, diphtheritic patches deep in the mucosal surface of the intestine and in some cases perforating the wall of the intestine causing peritonitis. A yolk culture heated at 60 C for

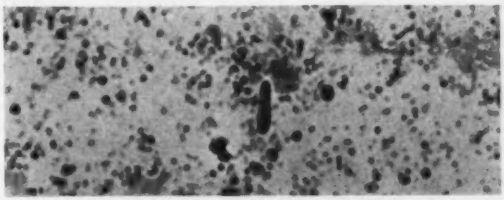

Fig. 4. Anaerobic bacillus containing subterminal spore from yolk culture stained with basic fuchsin. 2700×.

one hour, and passed twice in embryos was lethal when fed to quail. The bacillus, when serially transferred four times in thioglycollate medium, was non-infectious in one feeding trial with quail. However, when a fifth passage in thioglycollate was followed by one embryo passage, the harvested yolk was pathogenic for quail. Embryo isolates of the bacillus from two quail which died following feeding of yolk cultures have proved pathogenic to susceptible quail following oral inoculation. A yolk culture of the bacillus isolated from a chicken liver was pathogenic when fed to quail. Five day embryos are readily killed in 48 to 72 hours following yolk sac inoculation. Most ten day embryos survived when inoculated with the organism via the chorio-allantoic sac.

DISCUSSION

The organism described herein and that reported by Bass have several characteristics in common. Although Bass did not describe the morphology of his bacillus in detail, the writer

examined colored photos of the bacillus made by Bass. Both organisms have a similar morphology. Both organisms are anaerobic. The fact that Bass grew all of his cultures in artificial media could well explain their Gram negative tendency and failure to form spores. It has been our observation that spores are more abundantly produced in the yolk sac of embryos than in vitro culture and that cultures in artificial media are more readily decolorized with the Gram technique. Kirkpatrick[9] reported that streptomycin in the drinking water exerted a marked deterrent effect on the development and course of ulcerative enteritis in quail produced by experimental exposure. The organism described by us was sensitive to both streptomycin and penicillin as assayed by embryo mortality.

ACKNOWLEDGMENTS

I wish to express my appreciation to Mr. Earl Holm, superintendent of the Bureau of Game, New York State Conservation Department, Albany, New York, for his cooperation and generosity in making quail available for this study. Thanks are also due Mr. Richard Reynolds, manager of the Ithaca Game Farm, for the maintenance and supply of quail used in this study. I also wish to thank Dr. P. P. Levine, New York State Veterinary College, for his advice and assistance in this study and in the preparation of the manuscript.

SUMMARY

Two outbreaks of ulcerative enteritis in chickens have been described. Intestinal suspensions from dead chickens when fed to quail produced an ulcerative enteritis characteristic of "quail disease". From the blood and livers of these quail, a Gram positive, spore forming anaerobe was isolated in embryos. A bacillus with similar characteristics was isolated from the liver of a chicken which died with ulcerative enteritis. This organism failed to grow under aerobic conditions. In embryo assay, the organism was susceptible to penicillin and streptomycin. The bacillus resisted heating in a water bath for 2½ hours at 60 C. Yolk cultures of the bacillus killed quail on feeding in 2 to 7 days with liver and

intestinal lesions of ulcerative enteritis typical of "quail disease". The organism has been reisolated from quail which died following oral administration of yolk cultures. Pheasants, poults, and chicks did not become infected when fed yolk cultures of the organism. Cultures grown in vitro failed to infect quail when inoculated per os. Yolk from a single embryo passage following in vitro cultivation was infectious for quail.

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FIELD TRIALS WITH HYGROMYCIN AS AN ANTHELMINTIC IN POULTRY*

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IN recent years there have been many changes in management practices on commercial egg and breeder farms in the northeast. Most conspicuous are: 1. Depopulation is rarely, if ever, practiced. 2. Replacement birds are primarily confinement reared. 3. Several age groups are kept on the same farm. 4. More birds are kept in the same amount of space. These changes all presumably aid the poultryman in producing eggs more efficiently, yet they make disease and parasite

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control more difficult. In particular the control of *Capillaria obsignata* (*columbae*) has become more difficult. This communication reports the efficacy of Hygromycin (*Streptomyces hygroscopius* fermentation products, Lilly) against certain internal parasites of poultry.

C. obsignata (columbae) was first reported by Graybill[3] in 1924. He found this parasite present in very small numbers. Morgan[7] in 1932 found this parasite to be common in poultry in Herefortshire, England. Todd[10] in 1946 in a survey of parasites of chickens in Tennessee, reported the largest number from a single bird to be 11 while Morehouse[6] in 1944 reported 346 in a single bird. In Connecticut a survey of 218 flocks revealed a 67 percent incidence of infection with *C. obsignata*[3]. Angstrom *et al.*[1] indicated 1864 cases of capillariasis diagnosed in the diagnostic laboratories of the 13 northeastern states and three Canadian laboratories for 1958. Stubbs and Crawley[9] in 1922 described the ova of an intestinal capillarid that could have possibly been *C. obsignata.* Measurements they cited were more nearly those of *C. obsignata* than any of the other capillarids commonly found in poultry. Rietz[8] in 1927 found Kamala, Kamala plus soda, turpentine plus linseed oil, areca, and areca and soda to be of little value in the removal of *Capillaria sp.* He found single doses of carbon tetrachloride to be effective and also carbon tetrachloride repeated in 7 days to cause improvement in a flock where capillariasis had been diagnosed. Levine[5] in 1938 found single doses of carbon tetrachloride and tetrachloroethylene to be relatively ineffective. He also found tobacco dust (nicotine) in the ration at a two percent level to be ineffective in preventing infection. Up until the present time, no drug that could be used as a mass treatment has been reported to be of value. The author[2], found massive doses of piperazine (1700 milligrams per bird) to be of little value. Only 10 percent (20 of 200) of the adult capillarids were removed. Goldsby and Todd[4] list an effective anthelmintic as one that is "not toxic when fed continuously, palatable, effective against all species of internal parasites, and capable of reducing the reproductive capacity of the worm species to prevent

reinfestation". They also reported Hygromycin to fulfill their requirements as an anthelmintic in swine.

Three field trials were run to test the efficacy of Hygromycin against the internal parasites of poultry.

MATERIALS AND METHODS

Field Trial 1. A flock of 1970 White Leghorns (WL) kept in one pen and varying in age from 47 to 63 weeks was treated. Eight hundred of the same birds served as controls. Hygromycin was mixed with the regular egg-breeder mash and fed at the rate of 8 grams (8 million units) per ton of mash for 8 weeks. Eight hundred similar birds were kept as untreated controls in another pen. Complete feed consumption and egg production records were kept. One percent of the treated group were killed and necropsied prior to the feeding of any medicated ration. One percent of both treated and control groups were killed and necropsied after the medicated ration had been fed for 2, 4, 6, and 8 weeks. If a pathological alteration was noted at necropsy, the organ suspected was submitted for histopathological procedures. The intestine was opened longitudinally and the presence or absence of *Ascaridia galli* was recorded. The intestine was cut into short lengths and floated in 2 percent saline solution in petri dishes. These were examined under a dissecting microscope at 120 magnification and the presence or absence of *C. obsignata* was recorded.

Field Trial II. Six months later this trial was run on the same farm as trial I. The entire flock was fed Hygromycin mixed in the regular egg-breeder mash at the rate of 8 grams per ton (feed analysis showed actual level of Hygromycin was 7.98 grams per ton) for 12 weeks. This trial was designed primarily to detect possible toxicity. The 18,000 WL consisted of four different strains and varied in age from 29 to 86 weeks. Feed consumption and egg production records were kept throughout the trial. At the end of the 12 weeks feeding period, birds from each of the four buildings on the farm were necropsied.

One hour sedimentation rates and hematocrits were run

of fresh, whole, citrated blood from each of 20 birds. The liver, kidney, and spleen of 10 birds were examined histologically. The entire intestine was opened longitudinally and placed in 5 percent saline solution for one hour. The intestine and its contents were washed thoroughly with a fine spray of water onto a 100-mesh sieve. The material retained was examined and the *A. galli* and *C. obsignata* counted from each individual bird.

Field Trial III. 1100 sex link, cross chickens, 9 months old, were fed their regular commercial egg ration with 10 grams of Hygromycin per ton added. This ration was fed in a crumble form. Eleven birds were necropsied prior to treatment and 4, and 8 weeks after treatment was started. Intestines were processed as trial II. The ceca were processed in the same manner and *Heterakis gallinae* were counted. The liver, kidney and spleen of each necropsied bird were examined histologically.

RESULTS

Field Trial I. Table 1 shows the rate of infection with *A. galli* and *C. obsignata.* The rate of infection of *C. obsignata* in the treated birds was reduced from 100 percent to 5 percent of the total birds examined. The rate of infection in the controls increased from 62 to 75 percent of the total birds examined. The rate of infection of *A. galli* in the treated birds decreased from 23 to 0 percent while the control birds decreased from 75 to 25 percent. The egg production and feed consumption remained essentially the same throughout the trial. No other disease was noted in any of the birds examined.

Field Trial II. Hematocrits and one hour sedimentation rates on each of the 20 birds examined were within normal limits. Histologically the tissues from the 10 birds examined were normal. Egg production increased and feed consumption decreased in the flock as a whole during the trial. Of the 20 birds examined, one had 3 *A. galli* present and six had the following number of *C. obsignata* present, 1, 1, 1, 15, 18, 27, respectively. This flock was known to be heavily infected at the time field trial I was started. It is probable that some control of these parasites was obtained.

TABLE I

Rate of infection with *C. obsignata* and *A. galli*.
After treatment with 8 grams per ton Hygromycin. Field trial I.

Duration of treatment	Capillaria obsignata				Ascardia galli			
	Treated		Controls		Treated		Controls	
	No.*	%	No.*	%	No.*	%	No.*	%
None	21 of 21	100	—	—	5 of 21	23	—	—
2 wks.	13 of 18	72	5 of 8	62	7 of 18	39	6 of 8	75
4 wks.	12 of 23	52	7 of 8	88	1 of 23	4	5 of 8	62
6 wks.	8 of 26	30	6 of 8	75	2 of 24	8	4 of 8	50
8 wks.	1 of 20	5	6 of 8	75	0 of 0	0	2 of 8	25

* Indicated number of birds infected per number examined.

TABLE II

Number of *Capillaria obsignata* from 33 individual birds fed 10 grams Hygromycin per ton of feed. Field trial III.

	Prior to treatment	4 wks. medication	8 wks. medication
	0	0	0
	0	0	0
	37	0	0
	60	13	0
	60	13	0
	180	20	7
	217	20	12
	224	28	22
	232	69	25
	360	75	36
	510	450	52
Total	1880	688	154
Average per bird	170.9	62.5	14

TABLE III

Egg production and feed consumption of birds in field trial III

	% Production	Feed consumption*
Prior to treatment	39	40
8 wks. medication	52	33
5 wks. after cessation of treatment	64	29

* Pounds of feed per 100 birds per day.

Field Trial III. Table 2 shows the number of *C. obsignata* per bird examined throughout the trial. The 11 birds prior to treatment were all infected with *H. gallinae* with numbers from 10 to 215 (average 92). At 4 weeks and 8 weeks no *H. gallinae* were observed. Prior to treatment one bird had 15

A. galli. After 4 weeks of treatment no birds had *A. galli* and after 8 weeks of treatment, two birds had 2 and 4 *A. galli* respectively. After the 8 weeks' treatment, two birds had tapeworms—*Hymenolepis carioca.* Hematocrits and one hour sedimentation rates were all within normal limits. Tissues of three of the birds examined prior to treatment showed very slight lesions characteristic of avian infectious hepatitis. All others were considered normal. After 8 weeks of treatment, three birds showed slight lesions characteristic of avian infectious hepatitis. All other birds were considered normal. Table 3 shows a summary of the egg production and feed consumption records of the birds throughout the trial and for 5 weeks following the trial.

ACKNOWLEDGMENTS

The author wishes to thank Messrs. Parizek and Pirozok for preparing the histological sections and for assistance in preparing the hematological procedures.

SUMMARY

Three field trials were run to test the effectiveness of Hygromycin (Lilly) as an anthelmintic in laying hens. Field trial 1 indicated that this material was effective against both *C. obsignata (columbae)* and *A. galli* when fed at the rate of 8 grams per ton of feed for 8 weeks.

The results of field trial II indicated that Hygromycin did not adversely affect egg production or feed efficiency when fed at the rate of 8 grams per ton for 12 weeks.

Field trial III indicated partial control of *C. obsignata,* complete control of *H. gallinae.* The infection with *A. galli* was not severe enough to permit conclusions to be drawn.

The findings in these trials indicate that Hygromycin may have a place in the control of *A. galli, C. obsignata,* and *H. gallinae* in commercial poultry flocks.

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ABSTRACTS OF PART OF THE PAPERS PRESENTED AT THE 31st NORTHEASTERN CONFERENCE ON AVIAN DISEASES, JUNE 22-24, 1959, UNIVERSITY OF CONNECTICUT, STORRS, CONNECTICUT

The papers presented here in abstract form represent a part of the program. The remainder of the papers are presented in their entirety elsewhere in this issue.

PLAQUE FORMATION OF AVIAN ENCEPHALOMYELITIS VIRUS IN A CHICKEN EMBRYO KIDNEY CELL CULTURE SYSTEM*

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Plaque formation by avian encephalomyelitis virus in a chicken embryo kidney cell culture system was demonstrated. Plaques became recognizable on the 9th day of incubation. Their size increased gradually and reached a maximum on the 30th day of incubation when they measured 15 mm in diameter. The maximum size of the plaques was uniform at any given time. The plaques were circular with a sharp smooth edge. One ml of the seed virus contained 10^9 plaque forming units.

* Supported in part by funds from the American Poultry and Hatchery Federation.

SOME OBSERVATIONS ON AVIAN OSTEOPETROSIS*

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Observations were made on the clinical and pathological aspects of

avian osteopetrosis in Great Britain. The disease was found to be transmissible. It was experimentally transmitted following the intraperitoneal injection of day-old chicks with whole blood from donors which were active cases of osteopetrosis as judged by increased heat over the metatarsals and new-bone formation visible radiologically. Approximately 46% of injected birds developed the disease. The daily administration of cortisone acetate intramuscularly for 23 days after inoculation did not influence the incidence. Osteopetrosis was also transmitted following the intraperitoneal injection of day-old chicks with bone marrow suspension, unfiltered plasma and Seitz EK filtered plasma and by the oral administration of unfiltered plasma.

Osteopetrosis also developed in some birds which had been inoculated as 8 to 12 day old embryos by the intraamnion route using blood from affected birds. This suggested that the egg might be one mode of natural transmission. Further support for this has been provided by the appearance of the disease in the progeny of affected pullets which had been artificially inseminated with semen from affected males.

Histologically the primary lesion consists of hyperaemia followed by periosteal proliferation in the mid-shafts of the long bones. In advanced cases virtually the whole skeleton is affected including the pectoral and pelvic girdles, the digits and the skull.

DETECTION OF *SALMONELLA TYPHIMURIUM* INFECTION IN AN EGG-PRODUCING CHICKEN FLOCK

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In a recent *S. typhimurium* outbreak among patients in two state hospitals, unpasteurized eggnogs were suspected as the source of the infection. The eggs used in the preparation of the eggnogs were produced by the poultry flock on one of the hospital farms.

At the request of Dr. Robert A. MacCready, Massachusetts Department of Public Health, Jamaica Plain, Massachusetts, arrangements were made to test the poultry flock for typhimurium infection. On February 16 to 24, 1959, approximately two months after the hospital epidemic, a total of 5,050 chickens was tested with the tube agglutination test using a typhimurium antigen furnished by Dr. B. S. Pomeroy, College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota. Ninety-four reacting birds were detected of which nine of the stronger reactors were submitted to the laboratory for bacteriological examination. Lesions suggestive of salmonellosis were observed in several of the birds and *S. typhimurium* was isolated from two of the reactors. These cultures, as well as two cultures isolated from the patients, were typed as *S. typhimurium* by Dr. Philip R. Edwards, Communicable Disease Center, Chamblee, Georgia. Also these same cultures were submitted to Dr. E. S. Anderson, Central Public Health Laboratory, London, who reported that these cultures belong to phage type I, variation 5, of *S. typhimurium*. It was intimated that these findings supported the suspicion that the eggs used in the eggnogs and produced by the hospital flock were the source of the infection.

More recently a small number of reacting birds from the above flock has been submitted to the laboratory for further study. Eggs laid by these reactors yielded *S. typhimurium* on culture in one instance.

HATCHERY SANITATION

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A method of assessing the sanitary conditions of hatcheries by microbiological analysis is presented. Results of the analyses of more than one thousand samples from over one hundred and twenty hatcheries are discussed. The high microbial populations, including coliforms and *Aspergillus* spp. discovered by this type of analysis are alarming. The fact that this situation exists even though disinfectants and fumigants recommended for hatchery sanitation are used, demands a review of the efficacy of many such recommendations.

THE THERAPEUTIC VALUE OF TERRAMYCIN-TPA ON CRD

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At 8 weeks of age, 5500 White Rock replacement pullets, immune to infectious bronchitis and Newcastle disease, began to exhibit respiratory symptoms. Within 3 days, the symptoms had increased rapidly and the pathology was typical of chronic respiratory disease. At this time, 4300 birds were placed on a medicated feed containing 200 grams per ton of terramycin with 0.375 percent terephthalic acid. The remaining 1200 birds were kept as non-medicated controls.

The mortality during the next 11 days increased, and reached a total of 5.42 percent and 4.03 percent for the medicated and non-medicated group respectively. Because of this, all birds (including the non-medicated controls) were injected with an antibiotic. Terramycin in propylene glycol (75 mg) was injected subcutaneously into the non-medicated controls and 1800 birds of the feed medicated group. Dihydrostreptomycin (100 mg) was injected intramuscularly into 2500 birds that received medicated feed. The mortality for 2 weeks following terramycin injection was 5.63 percent for the non-medicated feed group and 2.11 percent for the medicated feed group. The mortality was 1.90 percent for the medicated feed group injected with dihydrostreptomycin.

Even though the mortality increased while the birds were receiving terramycin and TPA, the total mortality during the 4 week period was slightly less in the feed medicated group than in the controls. Terramycin and dihydrostreptomycin appeared to be comparable in value in controlling CRD in this trial.

STUDIES ON *PASTEURELLA ANATIPESTIFER* INFECTION IN WHITE PEKIN DUCKS

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A highly pathogenic strain of *Pasteurella anatipestifer* was isolated from white Pekin ducks that were brought to the Duck Disease Laboratory ot Eastport, New York. It resembled the organism described by Hendrickson and Hilbert in 1932 under the name *Pfeifferella anatipestfier*, (Hendrickson, J. M., and K. F. Hilbert. A new and serious septicemic disease of young ducks with a description of the causative organism, *pfeifferella anatipestifer*, N.S. Cornell Vet., *22*, 239-252, 1932.) The clinical picture and gross pathologic findings were identical to those

of the original description. The only difference was that this culture maintained the same high level of virulence when it was transferred in artificial media and when it was lyophylized, stored, and several months later inoculated into birds. The ideal route of infection was via the trachea and deaths were produced in 24 hours or less. It required 48 hours to produce deaths when the birds were injected intraperitoneally. Feeding trials were unsuccessful.

Pure cultures were reisolated routinely from the heart, liver, spleen, brain, and wing vein. The medium of choice was PPLO agar and PPLO broth fortified with Difco serum fraction. The cultures were always incubated under 10% CO_2.

Embryonated hens' eggs were killed in 24 hours when inoculated via the yolk sac with dilutions up to and including a 10^{-8} dilution of a broth culture. Mice were refractory to this organism when injected intraperitoneally and when injected intracerebrally.

A rabbit was immunized with a formalinized broth culture. A titer of 1:5,120 was demonstrated by the indirect hemagglutination test when the supernatant of a boiled culture was absorbed to duck red blood cells. Titers up to 1:80 were noted when immune duck sera were run with the antigen adsorbed to sheep cells and adsorbed to chicken cells, but no titers were seen when these same sera were run with treated duck cells.

SALMONELLA PULLORUM AND *SALMONELLA HEIDELBERG* TYPING RESULTS IN 1958

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Six strains of *Salmonella pullorum* and 13 strains of *Salmonella heidelberg*, isolated from diagnostic specimens and reactors, were typed using the modified ammonium sulfate method of Williams and Harris (Jour. Am. Vet. Med. Assoc., *127*, 133-136, 1955). The results are shown in the accompanying table.

Typing results, 1958

Salmonella pullorum			Salmonella heidelberg		
Specimen number	Source	Degree of clearing	Specimen number	Source	Degree of clearing
A61527	I	C	A59905	T	C
A78180	I	C	A66691	M	C
A79028	I	C	A66712	M	C
A79444	I	C	A72924*	M	C
A79811	I	C	A73635*	M	C
A86588	I	C	A73646*	M	C
			A73759	Mink	C
LEGEND: * = reactor; C = complete clearing; T = turkey; M = mature chicken; I = immature chicken.			A75454	M	C
			A82612*	M	C
			A82628	M	C
			A83282*	M	C
			A83894*	M	C
			A87619	M	C

Using this method, all strains typed were shown to contain only the XII_3 factor. Comparative serologic tests by the method of Edwards and Bruner (Cornell Vet., *36*, 318-324, 1946) also indicated only the XII_3 factor present in these strains. These and previous results have shown that the testing program using the standard conference antigen is giving satisfactory pullorum disease control in Connecticut.

REPORT OF THE COMMITTEE ON NOMENCLATURE AND REPORTING OF DISEASES NORTHEASTERN CONFERENCE ON AVIAN DISEASES JUNE 1959

The purpose of this report is to present information on the incidence of disease in the area encompassed by this Conference. Furthermore it is hoped that a nomenclature and system of reporting of diseases can be developed which may serve to standarize the reports from the various laboratories.

The statistics are based on the calendar year 1958 with the exception of those for Canada and Delaware which are based on a fiscal year. Each unit statistic represents a single case or consignment to the laboratory and therefore approximates an outbreak of disease on a premises. The figures are reported by states rather than by member laboratory. Statistics shown for Canada were compiled from those submitted by the Ontario Veterinary College, Guelph and the Regional Laboratories at Ridgetown and Kemptville.

The incidence of disease is reported for age groups which coincide roughly with the brooding, growing and adult life of the birds. A separate category indicates incidence of disease in species other than chickens. Whenever possible the identity of the species is indicated; T—turkey; Ph—pheasant; D—duck; Pkt—parakeet; Pgn—pigeon; Go—goose; Cra—crane; Qu—quail; Gu—guinea and Jay—bluejay. More than one species were combined and designated by letter O.

All infectious and contagious, parasitic and nutritional diseases plus such miscellaneous and unclassified conditions and diseases as may be of significance or interest are reported. However limitations of space and variations in systems of reporting by the various laboratories make it necessary to be somewhat arbitrary in preparing the report and some discrepancies and omissions are unavoidable.

Specific nomenclature is used wherever possible to indicate specific disease entities. However, for expediency some classifications are used in a generic sense and singleness or identity of etiology is not indicated. Ascaridiasis, coccidiosis and pox are examples. Nutritional diseases are classified according to clinical syndrome. Thus under the term rickets are included cases of improper calcification of bones whether due to avitaminosis D, a deficiency or imbalance of minerals as calcium or phosphorus, or an excess of sulfur in the diet. The statistics are presumed to be essentially accurate even though not all diagnoses are based upon the isolation or identification of the etiological agent.

Certain unavoidable discrepancies are indicated by letter which also appears by the statistic to which it applies.

a—figure includes all chickens regardless of age
b—figure includes all avian species
c—immune to Bronchitis
d—figure includes some tape and cecal worms
e—figure includes all forms of Leukosis

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State		Conn.	Del.	Maine	Mass.	N.H.	N.J.	N.Y.	No.C.	Penn.	R.I.	Vt.	Va.	W.Va.	Canada
Consignment Totals		1771	2391	1302	1868	2228	6866	6029	5106	5550	833	384	4414	1350	4618
Acariasis	1-5 Mos	a 4				1	b 34	6		a 37		a 1	5		
	+ 5 Mos			5	13	4		52	4				2	1	1
	Other				T-1			O-5	Ph-1	T-2			T-2		
Anatapestifer infection	Other							D-183		D-1					
Aneurism, dissecting	1-5 Mos														1
	Other	T-1		T-1	T-14			T-6		T-10					T-19
Ascaridiasis	0-4 Wks	a 39					b929	1	4	a 407		a 13	11	d 3	1
	1-5 Mos		26	20	7	3		73	350		1		112	d 18	88
	+ 5 Mos		5	74	21	37		395	33		4		74	d 10	231
	Other		T-6					O-4	O-69	O-8			T-14	dT-14	O-7
Aspergillosis	0-4 Wks	a 2	27		2	1	b 11	11	3	a 26		a 3	15	14	16
	1-5 Mos		4	2		2		16	85				1		8
	+ 5 Mos			2	1			6	4				1		3
	Other	GO-2	T-8	T-4	O-3	T-1		O-19	T-23	O-32			T-37	T-1	O-44
Avitaminosis A	0-4 Wks		1		6		b 23		2	a 9					6
	1-5 Mos							2	5		2		2		1
	+ 5 Mos				1			17					5		6
	Other				D-1					T-1			T-3		
Blue Comb Complex	0-4 Wks	a 14					b124		1	a 115		a 22	2	1	1
	1-5 Mos		11					6	13		1		66	4	11
	+ 5 Mos		5		9	1		48	26		8		104	5	28
	Other		T-5		T-2			O-8	T-27	T-8		T-1	T-338	T-11	T-11
Botulism (Limberneck)	1-5 Mos		6			1	b 28	13	11	a 8				1	
	+ 5 Mos		2		1				10				1		1
	Other		O-2					O-4	T-4	O-4					
Bronchitis, Infectious	0-4 Wks	a 16	12		8	c 6	b 81	5	7	a 125	2	a 3	9		32
	1-5 Mos		48	17	20	c102		38	90		13		16	2	49
	+ 5 Mos		6	3	8	c 44		58	33		4		3		74
Capillariasis	1-5 Mos	a 88		12	5	7	b717	22	34	a 284	2	a 12	3		45
	+ 5 Mos			91	75	125		170	7		8		12		124
	Other	T-1			T-2			O-3	O-2	O-8			T-3		O-2

State		Conn.	Del.	Maine	Mass.	N.H.	N.J.	N.Y.	No.C.	Penn.	R.I.	Vt.	Va.	W.Va.	Canada
Consignment Totals		177	2391	1302	1868	2228	6866	6029	5106	5550	833	384	4414	1350	4618
Cholera, fowl	0-4 Wks	a 6	1				b 109			a 123		a 17		1	
	1-5 Mos		6		1	1		4	1				3	31	2
	+ 5 Mos		12	18	11	17		37	82				13	1	21
	Other		O-8		D-3			D-124	T-4	O-20	Ph-1		T-68	T-23	T-11
Chronic Respiratory	0-4 Wks	a 268	45	1	9	73	b 712	35	18	a 531	2	a 17	54	153	77
Disease & Infectious	1-5 Mos		482	30	210	232		406	535		19		337	270	358
Sinusitis	+ 5 Mos		16	10	79	64		167	30		19		43	21	165
	Other	T-4	T-38	T-1	O-26	T-2		O-22	T-10	T-46			T-194	O-108	O-119
Coccidiosis	0-4 Wks	a 126	3	1	6	8	b1618	39	20	a 518	5	a 41	99	51	32
	1-5 Mos		119	55	121	108		546	736		57		286	99	359
	+ 5 Mos		3	21	47	36		212	47		7		31	3	113
	Other	O-9	T-18		O-23	O-2		O-112	O-42	O-37		T-2	O-236	O-43	O-89
Coryza, Infectious	0-4 Wks	a 4					b 80		1	a 16		a 3			2
	1-5 Mos				1			1	3		1		7		3
	+ 5 Mos							2	24		3		4		13
Encephalomalacia	0-4 Wks	a 16		3	3	1	b 2	8	7	a 7			11		
	1-5 Mos			3	4			2	23				1		
	Other								T-1						
Encephalomyelitis,	0-4 Wks	a 40	43	33	49	39	b 30	104	150	a 113	5	a 11	42	11	64
Avian	1-5 Mos		9	4	6	7		15	3				4	1	11
Encephalomyelitis,	1-5 Mos						b 2		1						
Equine	Other										Ph-1				
Enteritis,	0-4 Wks	a 3	10	1		3	b 307		5	a 132			50		65
unidentified	1-5 Mos		107	24	15	22		50	108		6		202	3	163
	+ 5 Mos		23	45	4	36		13	14		97		68		85
	Other	O-3	O-41	T-3	O-18			O-2	T-61	O-52	T-1		O-309		O-169
Enteritis, Ulcerative	0-4 Wks	a 19			1		b 46	2		a 2					5
	1-5 Mos				28	3		33	1						9
	+ 5 Mos				1			3							4
	Other				Qu-1			O-8	O-12	O-7			Qu-3		O-8
Erysipelas	+ 5 Mos				2		b 5	3	1	a 2					3
	Other	T-4	T-2		T-15	T-2		T-13	T-22	T-18	T-1	T-1	T-20	T-7	O-70
Hemorrhagic syndrome	0-4 Wks	a 23				1	b 319	5		a 181		a 2	17	1	14
	1-5 Mos		42	4	4	39		176	69				145	9	47
	+ 5 Mos		1			13		9	9				10	4	1
	Other	T-1						T-1		T-2			T-28	T-3	T-18

State		Conn.	Del.	Maine	Mass.	N.H.	N.J.	N.Y.	No.C.	Penn.	R.I.	Vt.	Va.	W.Va.	Canada
Consignment Totals		1771	2391	1302	1868	2228	6866	6029	5106	5550	833	384	4414	1350	4618
Hepatitis, Duck Virus	Other							D-247							
Hepatitis, Infectious Avian (Vibrionic)	0-4 Wks	a 114					b 164	3	4	a 73		a 3			1
	1-5 Mos		1		11	5		21	11				1		2
	+ 5 Mos				45	7		240	42						4
	Other	0-4				T-1									
Hepatitis, unidentified	1-5 Mos	a 2		15			b 341	20		a 22			1		2
	+ 5 Mos			31	2			61						5	23
	Other	0-1			0-3					T-2			T-2		T-1
Hexamitiasis	Other		T-2						T-1	0-3				T-7	
Histomoniasis	0-4 Wks	a 18					b 332	1		a 104		a 5	2	2	6
	1-5 Mos		10	8	14	65		203	167		1		35	8	80
	+ 5 Mos		2		4	6		64	17		2		8		18
	Other	0-7	T-43	T-4	T-16	T-7		0-32	0-123	0-62	T-1	T-2	0-96	T-43	0-121
Laryngotracheitis, Infectious	0-4 Wks	a 75	7				b 150			a 21		a 2	3		2
	1-5 Mos		80	9	6	10		2			4		21	1	6
	+ 5 Mos		5	35	3	2		5			10		15		10
Leucocytozoon infection	Other								T-10				T-1	T-8	
Leukosis Erythroblastic / Myeloblastic	1-5 Mos	a 2						4		a 6			1		1
	+ 5 Mos				1			15	2		2		1		1
Leukosis Neural	0-4 Wks	a 63	1		1		b1261	2		a 184		a 14	7	• 2	1
	1-5 Mos		130	29	89	e159		204	266		19		114	• 15	94
	+ 5 Mos		7	29	24	e213		114			3		32	• 30	36
	Other		T-2			eT-1									
Leukosis Ocular	1-5 Mos		9					9	2	a 39		a 1	14		6
	+ 5 Mos		13	2	9			69	16		3		10		13
	Other														T-1
Leukosis Osteopetrosis	1-5 Mos		1					9		a 9	2		3		
	+ 5 Mos			1	1			19	4				2		1
	Other							0-1							
Leukosis Visceral	0-4 Wks	a 89	1					1		a 483		a 36	1		
	1-5 Mos		123	19	34			119	40		8		99		63
	+ 5 Mos		37	100	138			520	387		170		163		325
	Other		T-6		T-3			T-1	T-4	0-5			T-3		T-5
Newcastle Disease	0-4 Wks	a 46	22	1			b 269	12	6	a 131	3	a 3	2	1	3
	1-5 Mos		96	2	3	4		44	37		7		11	1	
	+ 5 Mos		3		3	1		38	5		4		1		
	Other	0-3	T-3		T-1			Ph-1		T-2					

State		Conn.	Del.	Maine	Mass.	N.H.	N.J.	N.Y.	No.C.	Penn.	R.I.	Vt.	Va.	W.Va.	Canada
Consignment Totals		1771	2391	1302	1868	2228	6866	6029	5106	5550	833	384	4414	1350	4618
Riboflavin deficiency	0-4 Wks	a 3		2	4	1	b 6	2		a 7			5		
	1-5 Mos												4		
	Other							Ph-1		T-1					
Rickets & Ostecmalacia	0-4 Wks	a 3			1		b 11	6	5	a 31		a 1	12		
	1-5 Mos			7	2			16	18		2		6		1
	+ 5 Mos				4			30					3		3
	Other				0-3			0-4	T-2	0-4	T-1	T-1	T-1		
Synovitis, Infectious	0-4 Wks	a 63	1			2	b 62	2		a 120		a 3	3	1	1
	1-5 Mos		103	91	61	67		152	105		7		135	35	63
	+ 5 Mos		6	37	25	46		32	14				15	2	5
	Other	T-2	0-11		T-4	T-1		T-3	T-27	T-9		T-1	T-95	T-27	T-19
Tendon, ruptured gastrocnemius	1-5 Mos	a 10		3	3	4		12		a 12	2				
	+ 5 Mos			2	11	20		7			1			1	1
	Other									T-1					
Teniasis	1-5 Mos	a 3	6	5			b273	3	34	a 117		a 3	9		7
	+ 5 Mos		8	5	12	31		38	8		2		20		21
	Other							0-4	0-2	D-1					
Trichomoniasis	1-5 Mos						b 3	2	4	a 13					1
	+ 5 Mos							1							3
	Other	Ph-2	T-2		Pgm-3			T-2		0-6					T-5
Tuberculosis, Avian	1-5 Mos						b 1	3		a 4		a 1			
	+ 5 Mos				2			7					7		32
	Other				0-2									0-3	0-3
Tumors	0-4 Wks	a 1					b227	2		a 38		a 2			
	1-5 Mos			2	3	10		24							
	+ 5 Mos			6	11	12		129							6
	Other	0-4			0-3			0-6		0-8					0-2
Typhoid, Fowl	0-4 Wks						b 37		3	a 26			2	2	
	1-5 Mos		3						7				6		
	+ 5 Mos		3						50				9		9
	Other								T-6	T-1			T-1		

State		Conn.	Del.	Maine	Mass.	N.H.	N.J.	N.Y.	No.C.	Penn.	R.I.	Vt.	Va.	W.Va.	Canada
Consignment Totals		1771	2391	1302	1868	2228	6866	6029	5106	5550	833	384	4414	1350	4618
Omphalitis	0-4 Wks	a 20	26	33	16	81	b 91	97	137	a 66	5	a 7	13	18	21
	Other	T-2	T-2	0-3	T-2	T-3		0-69	0-13	0-23		T-3	T-2	T-3	T-7
Ornithosis & Psittacosis	Other							0-10		Pkt-3					
Parecolon infection	0-4 Wks	a 2													2
	1-5 Mos					1									4
	+ 5 Mos								1				1		4
	Other				Jay-1					T-2					0-3
Paratyphoid infection	0-4 Wks	a 9	1				b 16	6	17	a 11			3		2
	1-5 Mos		2					2	21				2		4
	+ 5 Mos			2	3	1		3	11		2		3		1
	Other				0-10	T-3		0-57	T-6	0-6			0-5	T-3	0-8
Pediculosis	1-5 Mos	a 1					b 320	4	7	a 181		a 2	5		6
	+ 5 Mos		2		32	2		142	14				20	1	15
	Other				0-3			0-5		0-3	T-1				
Peritonitis	0-4 Wks			1			b 572	5	1	a 104					2
	1-5 Mos		2	1		2		16					2		5
	+ 5 Mos		16	13	10	1		247	77				9		95
	Other				Cra-1			0-114		T-2			T-2		0-3
Perosis	0-4 Wks	a 1	1		2		b 2	9	1	a 15		a 2	2	1	1
	1-5 Mos				5	6		33	5		1		3	2	21
	+ 5 Mos					2		5	1				1		
	Other		T-1			T-1		0-7		0-5			T-5		T-6
Pox	0-4 Wks	a 9	2				b 162			a 54					
	1-5 Mos		4		1			12	7				5		2
	+ 5 Mos		5		10	3		30	4		4		13	2	3
	Other							0-7		0-3			T-9		
Pullorum Disease	0-4 Wks	a 6	6			1	b 7	1	35	a 37		a 4	15	8	20
	1-5 Mos							1					2		2
	+ 5 Mos			1	7	2		11	2				4	21	4
	Other							Gu-1							T-2
Respiratory infection (unidentified)	0-4 Wks		1	11	3	25	b1124	8		a 29			23	5	3
	1-5 Mos		2	147	3	36		15					37	2	4
	+ 5 Mos		2	35	10	4		29					12		1
	Other			T-2	0-6			0-6		T-1			T-8	T-1	0-29

REPORT ON THE PULLORUM DISEASE TESTING RESULTS FOR THE NORTHEASTERN CONFERENCE ON AVIAN DISEASES

The Northeastern Conference on Avian Diseases (formerly the Northeastern Conference of Laboratory Workers in Pullorum Disease Control) voted at its 21st Annual Meeting to publish a brief summary of pullorum testing data that were compiled for the past six years. The Conference has compiled annual summaries of pullorum testing data submitted by participating laboratories since the Conference was organized in 1927.

A special committee was assigned the task to collect and compile the data each year prior to the Annual Meeting of the Conference and distribute a summary of the results to the participating members of the group. Special commendation should be given to Mr. H. R. Baker, Delaware Department of Agriculture (now retired) who for many years rendered a very faithful and conscientious service in collecting and summarizing the annual testing reports of the Conference. The Delaware Department of Agriculture is continuing to collect the testing information for the Conference.

A complete file of annual testing summaries is in the possession of the Permanent Secretary of the Conference, and anyone seeking testing information prior to the six years published in this issue of the journal may communicate with the Secretary for further data.

It is the interest of the Conference to publish annual testing summaries compiled from reports submitted by the participating states in the group. It is hoped that this information will reveal what progress is being made in pullorum disease eradication and that it may serve as a stimulus to expedite the further elimination of the disease throughout the country.

In addition to the data listed in the six-year summaries, several fowl other than chickens and turkeys were tested. In a few instances reactors were reported and only occasionally was *Salmonella pullorum* isolated. It is apparent that fowl other than chickens and turkeys are not a natural host for pullorum disease. However, the testing of fowl other than chickens and turkeys should not be discontinued until the disease has been reduced further in the various states.

Submitted by:

H. Van Roekel, Permanent Secretary

Summary of Pullorum Disease Testing Data Reported by Northeastern Conference on Avian Diseases

	1953-54					1954-55					1955-56				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Conn.	203*	688,342	.078	187	670,484	175	688,363	.001	175	688,363	170	692,647	.038	168	692,573
	35**	8,973	.401	32	8,577	27	10,938	.009	25	10,216	30	12,806	.66	29	12,803
Del.	433	541,346	.019	405	526,721	442	525,623	.015	428	508,380	417	430,023	.036	414	422,433
	18	3,766	0	18	3,766	20	4,521	0	20	4,521	19	4,073	0	19	4,073
Maine	438	1,504,735	.0001	432	1,484,362	424	1,486,226	.017	418	1,454,155	385	1,547,034	0	379	1,544,445
	15	14,148	0	15	14,148	12	8,900	.268	11	7,940	8	6,380	0	7	5,279
Md.	1,403	532,779	.14	1,258	503,029	1,040	576,948	.037	999	503,972	921	388,960	.053	903	369,723
	76	20,981	.13	71	20,856	70	21,672	.15	67	18,293	40	14,013	.75	40	13,217
Mass.	338	1,178,090	.004	259	896,118	311	1,101,543	.1	205	811,875	285	1,077,449	.022	211	858,543
	60	26,272	0	39	21,364	51	25,825	0	33	20,194	44	26,688	0	29	23,059
N.H.	483	1,519,875	.048	460	1,523,409	392	1,362,555	.0004	383	1,341,503	371	1,239,223	0	366	1,224,907
	14	7,631	0	14	7,631	12	8,096	0	12	8,096	11	6,723	0	11	6,723
N.J.	539	995,779	.016	467	896,542	473	994,791	.023	430	846,253	440	995,027	.021	426	961,718
	33	8,966	0	33	8,956	35	9,684	0	33	9,130	30	7,240	0	30	7,235
N.C.	3,275	2,403,311	.04	3,062	2,387,527	3,633	2,169,753	.04	3,616	2,161,650	2,376	1,931,375	.008	2,302	1,922,175
	58	36,910	.22	38	32,910	70	42,384	.46	61	39,112	54	37,259	.34	41	32,003
N.Y.	349	774,302	.029	316	711,244	299	719,464	.003	281	685,380	256	696,219	.0004	232	626,577
	49	39,062	0	49	39,062	46	37,628	0	46	37,628	39	36,970	0	39	36,970
Nova Scotia	44	89,515	0	44	89,515	31	95,731	0	31	95,731	26	91,147	0	26	91,147
	9	1,318	0	9	1,318	9	1,845	0	9	1,845	8	1,647	.85	7	1,517
Ontario	1,987	1,029,621	.12	1,822	893,504	1,889	1,082,918	.31	1,709	911,908	2,318	1,074,113	.145	2,128	986,414
	141	45,767	.02	129	38,596	144	50,767	1.18	91	23,114	123	51,133	.31	106	37,682
Pa.	1,664	1,789,086	.48	1,005	1,013,917	1,548	1,855,477	.17	1,376	1,591,199	1,248	1,555,617	.112	1,129	1,445,732
	124	67,520	.14	78	49,226	113	61,111	.2	107	54,529	94	45,528	.03	52	31,546
R.I.	49	60,984	0	46	59,282	58	71,892	.008	55	71,123	43	47,500	0	42	47,230
	18	1,994	0	18	1,994	8	1,192	0	8	1,192	3	852	0	3	852
Vt.	279	251,200	.02	279	251,141	251	266,913	0	251	266,913	219	233,623	.0008	212	223,838
	19	5,874	0	19	5,874	20	5,584	0	20	5,584	14	5,590	0	14	5,590
Va.	2,128	1,061,365	.84	1,934	865,409	2,091	1,143,825	.082	2,020	1,060,767	1,676	1,003,853	.098	1,665	989,798
	542	294,616	.004	537	283,832	501	249,219	.002	498	243,179	582	264,398	.09	562	246,915
W. Va.	1,094	224,485	.05	1,081	213,753	1,022	246,674	.043	1,014	241,371	905	239,606	.02	905	234,582
	Turkey data not submitted					41	8,620	0	41	8,498	Turkey data not submitted				

1 - Number of flocks tested.
2 - Number of birds tested.
3 - Percent positive (based on number of birds tested).
4 - Number of pullorum-clean flocks.
5 - Number of pullorum-clean birds.
* - These figures under each state represent chickens.
** - These figures under each state represent turkeys.

Summary of Pullorum Disease Testing Data Reported by Northeastern Conference on Avian Diseases

	1956-57					1957-58					1958-59				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Conn.	189*	862,250	.003	185	861,420	144	730,964	.005	141	730,761	135	736,728	.004	132	736,470
	28**	11,671	.009	27	11,342	22	15,007	.03	21	14,666	18	11,178	0	17	11,176
Del.	343	497,126	.027	338	493,021	292	456,033	.03	279	454,711	311	467,102	.0006	310	466,066
	16	2,491	0	16	2,491	9	1,800	0	9	1,800	6	574	0	6	574
Maine	492	1,992,220	.0002	489	1,986,165	441	2,052,873	.0001	437	2,041,142	417	2,234,459	.0021	410	2,231,329
	8	6,043	0	7	6,038	4	4,362	0	4	4,362	4	2,499	0	4	2,499
Md.	957	479,060	.04	896	477,531	693	350,750	.115	683	328,329	621	326,349	.016	601	326,298
	46	21,007	.067	45	20,951	24	12,847	.17	23	11,744	19	13,222	.015	17	13,220
Mass.	272	1,093,056	.009	186	810,358	269	1,008,750	.0002	170	775,282	218	944,955	.0001	143	721,826
	41	22,775	0	26	18,499	26	15,985	0	20	12,581	24	18,014	0	15	14,266
N.H.	324	1,252,185	.002	302	1,205,521	255	981,557	0	246	962,080	231	1,031,004	0	222	1,016,024
	12	8,234	0	12	8,234	11	9,729	0	11	9,729	11	14,982	0	11	14,982
N.J.	389	905,429	.0064	365	859,537	287	795,873	.008	279	766,385	253	674,646	.02	243	663,847
	28	7,857	0	28	7,839	16	4,353	0	16	4,348	16	5,551	0	16	5,551
N.C.	2,716	2,344,794	.008	2,624	2,397,648	2,395	2,501,614	.004	2,365	2,463,604	2,496	3,020,677	.007	2,460	3,011,635
	65	59,800	.14	46	42,740	59	58,528	.16	48	53,711	58	64,146	.04	52	39,245
N.Y.	250	749,217	.0003	220	633,107	210	681,902	.0007	203	671,946	190	653,090	.0002	174	609,840
	38	33,342	0	38	33,342	29	31,039	0	29	31,039	26	23,796	0	26	23,796
Nova Scotia	27	90,583	0	27	90,583	25	66,452	0	25	66,452	28	79,649	0	28	79,649
	10	1,620	0	10	1,620	6	832	0	6	832	4	854	0	4	854
Ontario	2,601	1,316,030	.07	2,452	1,234,581	2,503	1,376,577	.031	2,406	1,337,765	2,371	1,398,777	.02	2,314	1,374,154
	113	57,821	.01	109	56,357	88	67,696	.01	87	65,037	151	104,761	0	151	104,761
Pa.	1,286	1,646,042	.07	1,210	1,518,011	1,121	1,546,536	.074	1,087	1,492,092	1,053	1,477,722	.09	1,033	1,445,289
	75	45,679	.008	67	43,554	69	37,103	.07	65	35,876	56	29,230	0	56	29,230
R.I.	29	33,433	0	27	32,777	36	47,983	0	32	46,429	34	45,866	0	31	29,332
	2	771	0	2	771	2	623	0	2	623	1	575	0	1	575
Vt.	227	255,507	.001	225	249,628	181	222,403	0	181	222,403	148	203,440	0	148	203,440
	13	4,760	0	13	4,760	13	4,357	0	13	4,357	5	4,103	0	5	4,103
Va.	1,819	1,269,459	.042	1,795	1,141,761	1,286	1,241,745	.03	1,360	1,112,821	1,445	1,136,947	.0226	1,320	1,136,689
	671	337,090	.009	669	317,775	453	271,603	.02	458	245,586	431	237,966	.0012	426	237,963
W. Va.	851	232,566	.005	843	228,466	732	203,132	.004	732	201,408	573	178,137	.0045	572	175,397
	31	8,720	0	31	8,539	26	12,586	0	26	12,391	34	16,066	0	34	15,626

1 - Number of flocks tested.
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4 - Number of pullorum-clean flocks.
5 - Number of pullorum-clean birds.
* - These figures under each state represent chickens.
** - These figures under each state represent turkeys.

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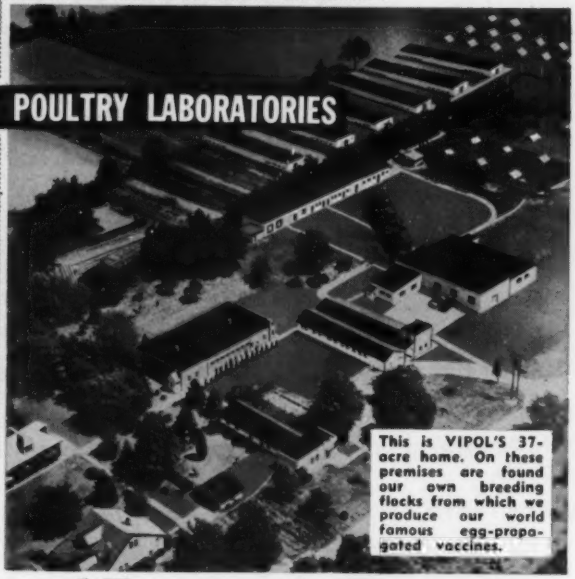

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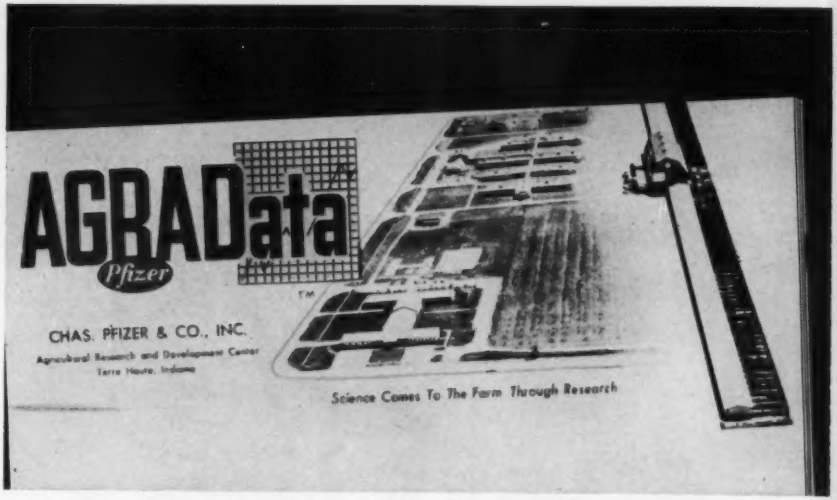

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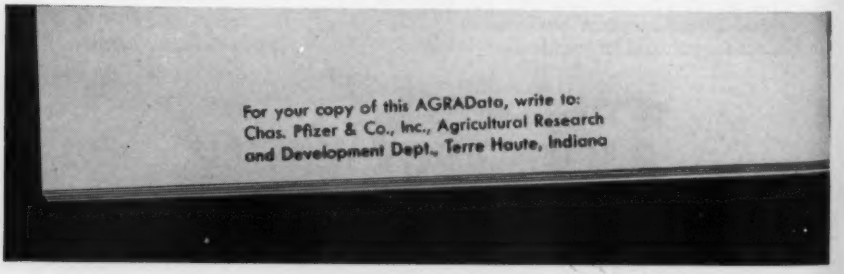
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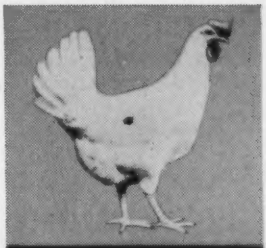

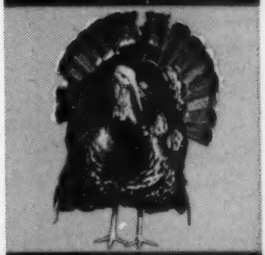

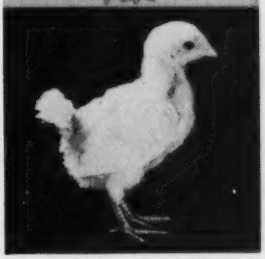

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GALLIMYCIN provides effective therapy for pleuropneumonia-like organisms, principal causative agents in over 80% of the bacterial infections involving respiratory disorders of poultry, PLUS treats many secondary organisms including Gram-positive and certain large viruses.

2. GALLIMYCIN Exerts Bactericidal Action

Few sensative organisms can survive GALLIMYCIN treatments. GALLIMYCIN is bactericidal in its action in contrast to the primarily bacteriostatic action of other leading antibiotics.

3. GALLIMYCIN Effective In Lower Doses

Lower approved doses of GALLIMYCIN reduce treatment costs as much as 25%. Approved dosage levels of ½-lb. of water-soluble GALLIMYCIN Poultry Formula will treat 50 gallons of water.

4. GALLIMYCIN Protects PPLO Infected Chick Embryos

Studies have shown this protection is accomplished with no apparent disturbance of egg production or hatchability.

GALLIMYCIN is also available in two feed forms, GALLIMYCIN-10 and GALLIMYCIN-TF. For more complete information, call or write: Veterinary Dept., Abbott Laboratories, North Chicago, Illinois.

Gallimycin
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MERCK & CO., Inc.
CHEMICAL DIVISION
RAHWAY, NEW JERSEY

specific

DUOSTREP® *is specific!* Combining equal parts of dihydrostreptomycin and streptomycin sulfates DUOSTREP is highly active against the pathogenic strains of PPLO often incriminated in outbreaks of infectious sinusitis in turkeys.

effective

DUOSTREP *is effective!* Unless the sinus is greatly extended, it is not necessary to drain exudate before injecting DUOSTREP. In most cases, one injection—at the rate of 0.4 cc. per sinus—relieves distended tissues ...minimizes the handling of affected flocks.

convenient

DUOSTREP *is convenient!* Supplied in 500 cc., rubber-capped bottles—enough to treat more than 1200 sinus cavities—DUOSTREP Injectable Solution requires no mixing...can be easily carried into brooding houses, breeding pens or range. (DUOSTREP can be stored for months under refrigeration with little or no loss in activity!)

low in cost

DUOSTREP *is low in cost!* In the treatment of infectious sinusitis, *because of its specific action in this disease,* DUOSTREP is more economical to use than broad-spectrum antibiotics. For specific, effective, convenient treatment of infections sinusitis, use or recommend low-cost DUOSTREP Injectable Solution.

for treatment of infectious sinusitis

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